

Yiqi Yangxue Qufeng Mixture alleviates knee osteoarthritis by modulating mitochondrial oxidative stress and suppressing ferroptosis via the PRKCA-MAPK/ERK axis

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Abstract

Background

Knee osteoarthritis (KOA) is one of the most common degenerative joint diseases globally, which significantly impairs patients' quality of life. Currently, effective approaches capable of reversing or significantly delaying disease progression are still lacking, and the long-term prognosis for patients remains suboptimal. This experiment aimed to validate the therapeutic efficacy of Yiqi Yangxue Qufeng Mixture (YQYXQF) in treating KOA and to elucidate the specific mechanisms through which YQYXQF alleviates KOA by restoring mitochondrial function.

Methods

The therapeutic efficacy of YQYXQF in treating KOA was systematically evaluated using a mouse model of knee osteoarthritis established via destabilization of medial meniscus (DMM) surgery. In vitro, the protective effects of YQYXQF on chondrocytes were evaluated in an IL-1 β -induced inflammatory microenvironment mimicking KOA. From an integrated analysis of single-cell omics and transcriptomics, Protein kinase C alpha (PRKCA) emerged as a candidate target for KOA therapy. Subsequent investigation of the LC-MS/MS-identified active components of YQYXQF was undertaken through network pharmacology and molecular dynamics simulations. Combined with in vitro and in vivo animal experiments validated the interaction between YQYXQF and PRKCA within the therapeutic mechanism of osteoarthritis.

Results

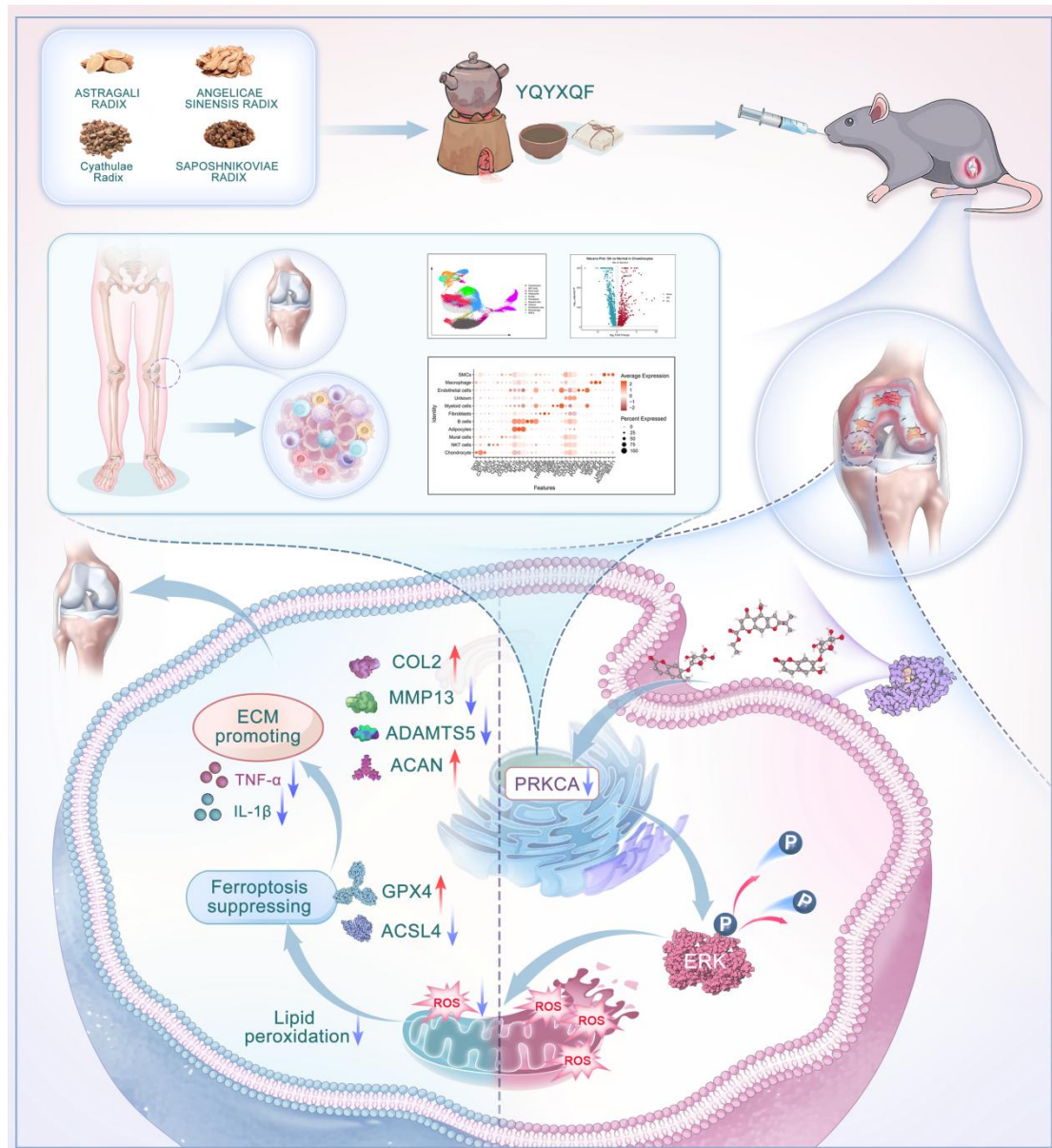
In vivo, YQYXQF significantly ameliorated knee joint injury induced by DMM surgery in mice. In vitro, YQYXQF treatment markedly improved mitochondrial morphology in chondrocytes under IL-1 β -simulated osteoarthritic conditions, alleviated mitochondrial oxidative stress, and reduced chondrocyte ferroptosis. Through the integrated application of Single-cell RNA-seq, Transcriptomics, and network pharmacology, the involvement of PRKCA in KOA progression was identified. Mechanistically, YQYXQF inhibited PRKCA expression, attenuated the phosphorylation-mediated activation of the MAPK/ERK signalling pathway, restored mitochondrial function, and mitigated mitochondrial oxidative stress and ferroptosis,

ultimately protecting chondrocytes and ameliorating osteoarthritis.

Conclusion

This study provides novel evidence that the traditional Chinese medicine (TCM) decoction YQYXQF exerts its biological effects by inhibiting PRKCA expression, thereby modulating the MAPK/ERK signalling pathway and restoring the function of the mitochondria. This ultimately leads to the alleviation of mitochondrial oxidative stress and ferroptosis in chondrocytes. These findings not only underscore the potential of the TCM compound YQYXQF as a therapeutic agent for KOA, but also provide a novel approach for targeting PRKCA/MAPK/ERK in the treatment of KOA.

Graphical abstract



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79 **Keywords:** KOA; Chondrocytes; TCM; PRKCA; Mitochondrial function; Ferroptosis

80 **Abbreviations**

81 BV/TV, Bone volume fraction; CCK-8, Cell counting kit-8; CETSA, Cell thermal

82 shift assay; DMM, Destabilization of the medial meniscus; GO, Gene ontology;

83 GSEA, Gene set enrichment analysis; H&E, Hematoxylin-Eosin staining; IF,

84 Immunofluorescence; IHC, Immunohistochemistry; IL-1 β , Interleukin-1 beta; KEGG,

85 Kyoto Encyclopedia of Genes and Genomes; KOA, Knee osteoarthritis; LC-MS/MS,

Liquid chromatography – tandem mass spectrometry; PMA, Phorbol 12-myristate 13-acetate; PRKCA, Protein kinase C alpha; ROS, Reactive oxygen species; RT-qPCR, Real-time quantitative PCR; siRNA, Small interfering RNA; SO-FG, Safranin-Fast Green staining; Tb.Sp, Trabecular separation; Tb.Th, Trabecular thickness; TCM, Traditional Chinese medicine; TEM, Transmission electron microscope; YQYXQF, Yiqi Yangxue Qufeng mixture.

Introduction

KOA is a degenerative joint disease for which epidemiological investigations suggest approximately 45% of the population may experience symptomatic episodes during their lifetime. [1]. From a pathological perspective, the onset of KOA results from the synergistic interplay of multiple factors. Elements such as malalignment of the knee joint, repetitive mechanical loading, obesity, altered bone density, muscle atrophy, and joint laxity converge to drive articular cartilage degeneration and synovitis through complex molecular cascades, creating a vicious cycle that mutually exacerbates both conditions [2,3]. As a critical load-bearing and shock-absorbing tissue, the cellular state of articular cartilage is crucial to the progression of KOA. [4]. Although current clinical pharmacological treatments can temporarily alleviate symptoms in KOA patients, effective interventions capable of reversing or significantly delaying disease progression are still lacking, leaving the long-term prognosis for patients suboptimal [5]. Thus, elucidating the molecular mechanisms underlying KOA progression and identifying novel therapeutic targets are crucial for addressing current clinical limitations and alleviating the associated patient and

societal burdens.

Elevated levels of mitochondrial oxidative stress and ferroptosis in KOA have been well-established by numerous studies as key drivers of the condition's progression. [6,7]. In patients with KOA, mechanical stress and inflammatory factors can cause dysfunction of the cell membrane. ROS is produced in large quantities, which leads to oxidative stress in the cell membrane [8]. ROS continuously attacks the mitochondrial membrane, causing an increase in the level of unsaturated fatty acids and initiating a series of lipid peroxidation reaction in the cell. Simultaneously, the activity of antioxidant enzymes decreased, failing to decompose the substantial accumulation of iron ions within the mitochondria due to disrupted iron metabolism. This process triggered ferroptosis; a form of programmed cell death that is iron-dependent. [9,10]. Therefore, the regulation of mitochondrial oxidative stress and ferroptosis is an important part of KOA treatment.

As a traditional medical practice with a history spanning millennia, TCM has garnered increasing recognition from various countries and regions, primarily due to its multi-faceted composition, numerous therapeutic targets, and low toxicity profile [11,12]. Recent studies have revealed that TCM plays a significant regulatory role in protecting mitochondrial structure and function, holding broad prospects for treating various diseases associated with mitochondrial dysfunction [13,14]. The therapeutic principles of TCM, characterized by holistic regulation, treatment based on syndrome differentiation, and simultaneously addressing both the root cause and clinical manifestations, demonstrate unique advantages in the prevention and management of

KOA. Based on TCM theoretical framework, the pathogenesis of KOA can be summarized as deficiency of both qi and blood constituting the root cause, while obstruction of muscles, tendons, and bones by wind-cold-dampness pathogens represents the secondary manifestations. Consequently, the core treatment principle should focus on supplementing qi and nourishing blood, dispelling wind and unblocking collaterals. The YQYXQF (composition: Astragali Radix, Angelicae Sinensis Radix, Cyathulae Radix, Saposhnikoviae Radix) aligns with the syndrome differentiation-based therapeutic approach for this condition. It is now widely applied in the clinical management of KOA and has established a relatively standardized treatment protocol. However, current understanding of this herbal mixture remains confined to its therapeutic efficacy in treating KOA, making it imperative to conduct a systematic analysis of its underlying mechanisms of action.

This study employed a systematic and integrative analytical strategy. First, single-cell data mining was used to identify PRKCA as the starting node. Subsequently, integrated analysis of transcriptomics and network pharmacology predicted that PRKCA is a direct target of YQYXQF in the treatment of KOA. PRKCA knockdown experiments and a series of rescue experiments then fully revealed the important role of the PRKCA–MAPK/ERK signalling axis in modulating chondrocyte mitochondrial oxidative stress and ferroptosis, thereby influencing KOA progression. Based on multi-level evidence, we have for the first time established a complete mechanistic chain demonstrating that YQYXQF delays KOA progression by affecting the PRKCA–MAPK/ERK signalling axis, inhibiting mitochondrial oxidative

stress, and alleviating ferroptosis.

Materials and methods

Materials

COL2 (Cat#: A1560) and GPX4 (Cat#: A1933) were purchased from ABclonal (Wuhan, China). MMP13 (Cat#: Ab39012) and IL-1 β (Cat#: Ab254360) were obtained from abcam (Shanghai, China). ACAN (Cat#: bs-11655R) and ADAMTS5 (Cat#: bs-3573R) were sourced from Bioss Biotechnology (Beijing, China). SOD2 (Cat#: ET1701-54) was supplied by Huabio (Hangzhou, China). PRKCA (Cat#: 21991-1-AP) was procured from Proteintech (Wuhan, China). The antibodies against ERK (Cat#: CST 4695) and phospho-ERK (Cat#: CST 4370) were sourced from Cell Signaling Technology (CST, Shanghai, China). PMA (HY-18739) was purchased from MCE (Shanghai, China). The MDA assay kit (S0131S), JC-1 mitochondrial membrane potential assay kit (C2006), reactive oxygen species assay kit (S0033S), ATP assay kit (S0027), BDPY 581/591 C11 (S0043S), and Erastin (SC0224) were all purchased from Beyotime Biotechnology (Shanghai, China). si-PRKCA and si-NC were purchased from GenePharma (Shanghai, China). YQYXQF (Zhejiang Drug Manufacturing Approval No.: Z20100036) is a standardized hospital preparation manufactured from Astragali Radix (27 g), Angelicae Sinensis Radix (27 g), Cyathulae Radix (27 g), and Saposhnikoviae Radix (13.5 g) following standardized procedures. It was provided by Hangzhou Hospital of Traditional Chinese Medicine (Hangzhou, China).

LC-MS/MS

The chemical composition of the sample was analyzed using ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry. The Chinese medicinal decoction to be tested, blank serum, and drug-containing serum were subjected to component separation using an Acquity CORTECS UPLC T3 column (2.4 × 100 mm, 1.6 μm), followed by detection via electrospray ionization (ESI) in both ESI⁺ and ESI⁻ modes. The results were analyzed using Unifi 1.8.0 software and visualized with Origin.

Animal experiment

Approval for the animal experiments conducted in this study was granted by the Animal Ethics Committee of Zhejiang Chinese Medical University (June 2025) (Approval No.: 20250623-16). Male C57BL/6 mice (8 weeks old) were purchased from the Animal Center of Zhejiang Chinese Medical University (Hangzhou, China), all of which showed generally similar health status and parameters suitable for the experimental requirements. All mice were housed in the specific pathogen-free animal facility of Zhejiang Chinese Medical University, maintained under controlled temperature and humidity conditions with a 12-hour light/dark cycle, and provided with ad libitum access to food and water. The mice were randomly divided into five groups, with ten mice per group. According to the literature, a mouse model of knee osteoarthritis was established using the DMM surgery [15]. In brief, after anaesthetising and disinfecting the mouse, a longitudinal incision was made along the anteromedial aspect of the knee joint to expose the patella. Following patellar dislocation and knee flexion, the medial meniscotibial ligament was identified and

transected, and then the patella was reduced and the wound was sutured in layers. Based on the conventional principle of dose conversion according to body surface area between humans and animals, taking into account the gavage volume limitation and tolerance status in mice during the animal experiments, the final intragastric dose selected was 16.6 g of crude drug per kilogram of mouse body weight per day. One week after modeling, the mice were divided into five groups: the CON group, the model group (DMM surgery + distilled water by gavage), the DMM surgery + low-dose YQYXQF group (4.15 g/kg/day), the DMM surgery + medium-dose YQYXQF group (8.3 g/kg/day), and the DMM surgery + high-dose YQYXQF group (16.6 g/kg/day). Eight weeks after the initiation of herbal gavage, mice from all groups underwent behavioral tests, including the observation of ambulatory movement and the measurement of knee joint samples. The knee joint samples were then fixed in 4% paraformaldehyde for two days and subjected to micro-CT scanning (Skyscan 1176, Bruker μ CT, Kontich, Belgium).

Hematoxylin-Eosin (H&E) staining and Safranin-Fast Green (SO-FG) staining

The samples were sequentially decalcified, dehydrated, embedded in paraffin, and sectioned into 4- μ m-thick slices. Sections from each group were deparaffinized and rehydrated. For H&E staining, the sections were stained with hematoxylin solution for 3 minutes, differentiated in hydrochloric acid-alcohol for 3 seconds, blued in ammonia water for 15 seconds, and then counterstained with eosin for 90 seconds. Osteoarthritis severity was graded for each sample using the OARSI scoring system. For SO-FG staining, the sections were stained with fast green for 5 minutes, rinsed

briefly in weak acid solution for 10 seconds and air-dried, stained with safranin O for 90 seconds, followed by dehydration and mounting.

Immunocytochemistry (IHC)

Following deparaffinization and rehydration, sections from each group were permeabilized with 0.3% Triton X-100 for 20 minutes. Antigen retrieval was then performed using citrate buffer at 65 degrees Celsius for 4 hours. After application of a 5% BSA block for 30 minutes, the sections were treated with correctly diluted primary antibodies for an overnight incubation at 4 degrees Celsius. The following day, an HRP-conjugated secondary antibody was applied and incubated at room temperature for 20 minutes. Color development was carried out using a DAB substrate, followed by counterstaining with hematoxylin for 20 seconds. Finally, the results were analyzed using ImageJ software.

Cell culture

Primary chondrocytes were isolated from the knee joints of 3-day-old male C57BL/6 mice under sterile conditions. The cartilage tissues were washed three times with PBS and then digested with 0.25% collagenase P (Roche, Shanghai, China) for 2 hours in a constant-temperature cell culture incubator at 37 degrees Celsius with 5% carbon dioxide. The digested cell suspension was subsequently filtered through a cell strainer into a 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes. After discarding the supernatant, the cells were washed once with PBS. The cell pellet was resuspended in F12 medium supplemented with 10% premium fetal bovine serum and 1% penicillin-streptomycin, transferred to a sterile culture dish, and P1 generation

cells were used for subsequent experiments.

To simulate knee osteoarthritis, chondrocytes were treated with IL-1 β (10 ng/ml) for intervention [16]. To investigate the mechanism of YQYXQF on KOA in vitro, 30 male SD rats with an average body weight of 220 g and in good health were purchased from the Animal Center of Zhejiang Chinese Medical University (Hangzhou, China). The rats were randomly divided into two groups: one group received intragastric administration of YQYXQF, while the other group received an equal volume of distilled water, to obtain drug-containing serum and blank serum, respectively. The Chinese medicinal decoction group was administered continuously for seven days at a dose of 9.7 g/kg/day. Approximately two hours after the final intragastric administration, rats in both groups were anesthetized, and blood samples were collected. The blood samples were centrifuged at 4000 rcf for 20 minutes to separate the upper serum layer, followed by complement inactivation. The serum was then filtered through a 0.22 μ m filter under sterile conditions and stored at -80 degrees Celsius. For cell intervention with serum, the CCK-8 assay was used to measure cell viability in order to determine the appropriate serum concentration for intervention [17]. Subsequently, primary chondrocytes were divided into the following groups: a control group, a model plus blank serum group, a model plus low-dose drug-containing serum group (2%), a model plus medium-dose drug-containing serum group (5%), and a model plus high-dose drug-containing serum group (10%). PRKCA was knocked down using small interfering RNA (siRNA) to study its impact on osteoarthritis. The ERK agonist Ceramide C6 was employed to activate ERK

phosphorylation, thereby investigating the mechanism of PRKCA's effect on ERK [18,19].

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using an RNA extraction kit (TransGen Biotech, Beijing, China). The concentration of RNA from each group was measured and quantified using a NanoDrop 2000 spectrophotometer, followed by reverse transcription into cDNA and subsequent RT-qPCR. β -Actin was used as the endogenous reference gene, and the expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences used are provided in the Supplementary Materials (Table S1).

Immunofluorescence (IF)

Primary mouse chondrocytes cultured in confocal dishes were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.3% Triton X-100 for 20 minutes, and then blocked with 5% BSA for 40 minutes. Subsequently, the cells were incubated with primary antibodies overnight at 4 degrees Celsius. Incubations were performed at room temperature in the dark on the following day: first with an Alexa Fluor® 594 dyes (abcam, ab150080) for 1 hour, and subsequently with DAPI for 10 minutes. Fluorescence images were acquired using a confocal microscope (ZEISS LSM880, Baden-Württemberg, Germany). The fluorescence intensity was analyzed and quantified using ImageJ software.

Western blotting

Total protein was extracted on ice using RIPA lysis buffer. Protein quantification

and concentration normalisation were performed using the BCA. Subsequently, the proteins were separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk at room temperature for 1 hour. Subsequently, it was incubated with diluted primary antibodies at 4 degrees Celsius overnight, followed by incubation with a rabbit secondary antibody (Abclonal AS014, Wuhan, China) diluted at 1:10,000 at room temperature for 1 hour. Protein bands were visualized using Image Lab software, and their band intensity was quantified using ImageJ software. β -Actin was used as a loading control for normalization.

Transmission electron microscope (TEM)

To observe the effect of YQYXQF on mitochondrial morphology, chondrocytes from each group were digested with trypsin for 1 minute. The harvested cells were then fixed with 2.5% glutaraldehyde and processed into ultrathin sections. Mitochondrial morphology was examined and imaged using a transmission electron microscope (Hitachi H-7650, Tokyo, Japan).

Mitochondrial membrane potential assay

Mitochondrial membrane potential levels in nucleus pulposus cells from each group were assessed using the JC-1 probe and laser confocal microscopy. Briefly, cells were seeded at an appropriate density in confocal dishes and allowed to adhere for 12 hours, followed by the designated interventions. A JC-1 working solution was prepared by mixing the JC-1 fluorescent dye, JC-1 staining buffer, and PBS at a ratio of 0.025:1:4. The JC-1 working solution was then added to the culture dishes and

incubated at 37 degrees Celsius for 20 minutes. After incubation, the cells were washed twice with JC-1 staining buffer (1X), and laser confocal images were acquired within one hour.

Lipid peroxidation MDA assay

The cells from each group were lysed using RIPA buffer and centrifuged at 12,000 g for 10 minutes. The resulting supernatant from each group was collected as the protein sample for MDA detection. The assay was performed using an Lipid Peroxidation MDA Assay Kit. Into a centrifuge tube, 0.1 ml of the protein sample was mixed with 0.2 ml of the MDA working solution; the mixture was then heated at 99°C in a heating block for 15 minutes. Following the reaction, the mixture was gradually cooled to room temperature in a water bath and spun at $1000 \times g$ for 10 minutes. From this, a 200 μ l aliquot of the supernatant from each sample was aliquoted into a 96-well plate. Absorbance was finally determined at 532 nm using a microplate reader. Additionally, the protein concentration of each sample was quantified using the BCA method. The MDA content (in nmol/mg protein) was determined based on the corresponding standard curve.

ROS assay

The intracellular ROS level in chondrocytes was assessed using the DCFH-DA probe, with detection performed by either inverted fluorescence microscopy or flow cytometry. Briefly, cells were seeded at an appropriate density in 6-well plates and allowed to adhere for 12 hours. Following the required experimental interventions, the cells were treated with DCFH-DA solution for 20 minutes. After washing three times

with PBS, the cells were trypsinized and analyzed within one hour using a flow cytometer (CytoFlex S, Beckman, Shanghai, China). The ROS levels in each group were quantified and analyzed using CytExpert.

BDPY C11 assay

Cells were washed with PBS, incubated with BDPY C11 working solution in a 37 degrees Celsius incubator for 20 minutes, washed twice with PBS, and then visualised using a confocal microscope.

ATP assay

Cells were lysed on ice using the ATP lysis buffer provided in the kit. The lysates were centrifuged at $12,000 \times g$ for 5 minutes at 4 degrees Celsius, and the supernatants were collected for further analysis. A volume of 100 μ l of ATP working solution was added to each well and incubated at room temperature for 3 minutes to deplete the background. Subsequently, 20 μ l of the prepared cell lysate was added to each well, and the luminescence intensity was measured using a luminometer. After protein quantification by the BCA method, the relative ATP content in each group was normalised to the control group.

Single-cell RNA-seq

The single-cell RNA sequencing data (GSE169454) used in this study were obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The 10x data for each sample were read using the Read10X function, and a Seurat object was constructed via CreateSeuratObject, ensuring data integrity and uniqueness. During quality control, cells with fewer than 300 or more than 5000 detected genes, or with a

mitochondrial gene percentage exceeding 10%, were filtered out. The data were then normalized and corrected for batch effects. Dimensionality reduction was performed using PCA and UMAP. Cell clustering was conducted with the FindClusters function, selecting a resolution of 0.5 as the optimal clustering criterion. Differential expression analysis was carried out using FindAllMarkers to identify marker genes for each cell population. The cellular distribution across different groups and the differentially expressed genes were visualized using UMAP plots and DotPlots. For differential expression analysis between specific groups, the FindMarkers function was applied with filtering thresholds set at a p-value < 0.05 and a log₂ fold change (log₂FC) > 0.25. GO, KEGG, and GSEA analyses were applied to identify potential pathways that may be involved in gene expression changes during KOA pathogenesis.

Transcriptomics

The groups were divided into CON group, MOD group, and YQYXQF group (n=4). Following three washes with PBS, total RNA was extracted from the chondrocyte samples using TRIzol reagent. The purity and integrity of the RNA were then verified, and all samples were uniformly quantified. The transcriptome library was constructed using the VAHTS Universal V10 RNA-seq Library Prep Kit (Premixed Version). Following sequencing on the Illumina NovaSeq 6000 platform, raw FASTQ data underwent quality control with fastp software to produce clean reads. Subsequent alignment to the reference genome was carried out with HISAT2, followed by quantification of gene expression levels as FPKM. Read counts for each gene were then obtained using HTSeq-count. Differential expression analysis

was performed using DESeq2. Genes meeting the thresholds of a q-value < 0.05 and a fold change > 2 or < 0.5 were defined as differentially expressed genes (DEGs) in this study. Subsequently, various analyses, including GO, KEGG, and GSEA, were conducted on the DEGs using R (v 3.2.0). The dataset supporting the conclusions of this article is included within the article and its additional file (Table S2).

Network pharmacology, molecular docking and molecular dynamics simulation

For the network pharmacology analysis, the active compounds were submitted to the SwissTargetPrediction database (<http://www.swisstargetprediction.ch/>) to predict potential therapeutic targets of the main active components in YQYXQF for KOA. Disease-related targets of KOA were retrieved from the GeneCards and OMIM databases. The intersection between the compound-predicted targets and the disease-related targets was considered as potential therapeutic targets. These potential targets were then imported into the STRING database to construct a protein-protein interaction (PPI) network, which was subsequently visualized using Cytoscape 3.8.0. For molecular docking, the protein structure of PRKCA (PDB ID: 4RA4) was obtained from the RCSB website, and information on the candidate compounds was downloaded from PubChem. Global molecular docking was then performed using AutoDock Vina software (exhaustiveness = 16, num_modes = 30). The resulting complex conformation with the lowest calculated binding free energy was selected as the initial structure for subsequent molecular dynamics simulations. Conventional molecular dynamics simulations were performed using the GROMACS software. After system construction, energy minimization was performed using 50,000 steps of

steepest descent, followed by system equilibration in two stages: 100 ps of NVT and 100 ps of NPT. The temperature was maintained at 300 K using the V-rescale thermostat algorithm, and the pressure was controlled at 1 bar using the Parrinello–Rahman barostat. After the equilibration phase, a 100 ns production simulation was conducted with all positional restraints removed. The trajectory was integrated with a time step of 2 fs, and long-range electrostatic interactions were accurately treated using the Particle Mesh Ewald (PME) method. Trajectories were saved every 10 ps, resulting in a total of 10,000 frames for subsequent analysis. In addition, stable trajectories were extracted from the late stage of the simulation (90–100 ns interval). The binding free energy of the ligand–protein complex was calculated using the gmx_MMPBSA tool, while trajectory analysis and visualization were performed using MolStar software.

Statistical analysis

Quantitative data are presented as mean±standard deviation (mean±SD). GraphPad Prism software was used for graph generation and quantitative data analysis. Unless otherwise specified, comparisons between two groups were analysed using Student's t-test, while comparisons among multiple groups were performed using one-way analysis of variance. A p-value < 0.05 was considered statistically significant. In vivo experiments were repeated at least five times, and in vitro experiments were repeated at least three times.

Results

YQYXQF alleviates cartilage damage in the mouse knee joint induced by DMM

surgery

To investigate the capacity of YQYXQF in slowing OA progression, a mouse model of osteoarthritis was generated via DMM surgery. Oral administration of YQYXQF was initiated 7- days post-surgery and continued for eight weeks (Fig. 1A). Eight weeks later, an open field test was conducted on mice from each group. Statistical analysis of the total exploration distance per unit time showed a significant decrease in the model group, whereas YQYXQF treatment markedly restored the exploration distance (Fig. 1B-C). Furthermore, the model group exhibited a marked decrease in the proportion of movement within the central zone, a change that was substantially reversed following YQYXQF administration. (Fig. 1B). Gait behavioural analysis was performed on mice from all groups (Fig. 1D). Relative to controls, alterations in the model group included an elevated swing time and diminished paw area, stride, and stride length. However, following YQYXQF treatment, values for swing markedly decreased, whereas paw area, stride, and stride length showed significant increases (Fig. 1E). Micro-CT scanning analysis was performed on the knee joints of mice from each group. At 8 weeks after DMM surgery, the model group showed a significant increase in bone volume fraction (BV/TV) and trabecular thickness (Tb.Th), along with a significant decrease in trabecular separation (Tb.Sp). YQYXQF treatment improved all these parameters. Micro-CT images further demonstrated that YQYXQF administration significantly alleviated DMM-induced subchondral bone sclerosis, markedly reduced osteophyte formation, and resulted in a smoother articular surface (Fig. 1F-I). Using H&E and SO-FG staining, we observed

severe cartilage damage in the model group, characterized by abnormal thinning of the cartilage layer and an irregular, fissured surface, relative to the normal group. YQYXQF treatment partially ameliorated the surface damage and improved cartilage thickness (Fig. 1J). Relative to the normal group, the OARSI score was markedly elevated in the model group, as assessed using the established OARSI criteria. However, YQYXQF treatment resulted in a significant reduction in the OARSI score (Fig. 1K). Additionally, SO-FG staining indicated that during disease advancement, the cartilage layer showed signs of fibrosis and fissuring, along with marked degradation of the red-stained collagen matrix. YQYXQF treatment partially restored the integrity of the cartilage layer and reduced matrix degradation (Fig. 1L). Collectively, these experimental results demonstrate that intervention with YQYXQF effectively attenuates cartilage degradation and delays the progression of osteoarthritis.

YQYXQF protects chondrocyte extracellular matrix via inhibiting mitochondrial oxidative stress and ferroptosis

For in vitro mechanistic studies, we isolated primary chondrocytes from wild-type C57BL/6 J mice and prepared YQYXQF-containing serum by orally dosing SD rats (weighing 220 g $\pm$ 20 g) with the formula daily for one week. The optimal concentration for high-dose serum intervention was determined to be 10% using the CCK-8 assay (Fig. S1A). TEM was used to observe ultrastructural changes in chondrocyte mitochondria (Fig. 2A). TEM revealed that, relative to the control group, mitochondria in the model group exhibited marked swelling along with atrophy,

460 fragmentation, dissolution, or even loss of cristae. These pathological alterations were
461 partially ameliorated following YQYXQF treatment. Mitochondrial oxidative stress
462 can impair mitochondrial membrane potential (MMP), leading to a decrease in the
463 ratio of JC-1 aggregates (red fluorescence) to monomers (green fluorescence).
464 Detection using the JC-1 Mitochondrial Membrane Potential Assay Kit showed that
465 YQYXQF treatment partially restored MMP levels (Fig. 2B-C). Confocal imaging
466 using an ROS assay kit showed that YQYXQF treatment partially alleviated ROS
467 accumulation (Fig. 2D-E). This observation was further corroborated by flow
468 cytometry analysis of ROS, where both the percentage of ROS-positive cells and the
469 median fluorescence intensity were significantly reduced following YQYXQF
470 treatment (Fig. S1B-D). In addition, mitochondrial ATP levels were restored following
471 YQYXQF treatment (S1E). MDA and BDPY C11 assays further showed that
472 YQYXQF treatment reduced MDA content and alleviated lipid peroxidation levels
473 (Fig. 2F, S1F-G). Confocal fluorescence imaging of GPX4, COL2, and MMP13
474 revealed that YQYXQF treatment reversed ferroptosis. The expression of COL2, an
475 anabolic marker of the extracellular matrix (ECM), was significantly upregulated,
476 while the level of MMP13, a catabolic marker of the ECM, was markedly
477 downregulated (Fig. 2G-H, S1H-J). Western blot analysis of key mitochondrial and
478 cartilage matrix-related proteins under IL-1 β -induced OA-like conditions
479 revealed marked reductions in the expression of SOD2 (reflecting mitochondrial
480 function) and the anabolic markers COL2 and ACAN. In contrast, a concurrent
481 upregulation was observed for the catabolic markers MMP13 and ADAMTS5.

YQYXQF treatment reversed these changes (Fig. 2I–J). Furthermore, the results of RT-qPCR confirmed the previously observed phenomena, and intervention with the ferroptosis agonist Erastin weakened the therapeutic effect of YQYXQF (Fig. 2K, S1K–M). These findings suggest that YQYXQF promotes chondrocyte anabolism and suppresses catabolism in vitro by inhibiting mitochondrial oxidative stress and ferroptosis.

Single-cell RNA-seq and Transcriptomics suggest PRKCA as a potential therapeutic target of YQYXQF

Through re-analysis of public single-cell transcriptomic data, we conducted a comprehensive investigation of the pathological changes associated with the progression of knee osteoarthritis (KOA) (Fig. 3A). Initially, raw data (Normal = 3; OA = 4) were downloaded from the GEO dataset GSE169454 and subjected to rigorous quality control (Fig. 3B). After stringent cell-based clustering, eleven distinct cell types were identified (Fig. 3C). Differential gene expression analysis of chondrocytes revealed that the expression of PRKCA was significantly elevated in chondrocytes from KOA patients (Fig. 3D, S2A). GSEA indicated that the MAPK signaling pathway was aberrantly activated within chondrocytes (Fig. 3E). To validate the results of single-cell sequencing, we examined the expression of PRKCA in cartilage tissue and cells in the model group. RT-qPCR confirmed that at the gene expression level, PRKCA was significantly upregulated upon IL-1 β stimulation (Fig. 3F). Immunofluorescence images showed that PRKCA fluorescence intensity was markedly increased following IL-1 β stimulation (Fig. 3G–H). Immunohistochemistry

data further corroborated this finding in vivo (Fig. 3I–J).

Subsequently, transcriptome sequencing was performed on mouse primary chondrocytes (Fig. 3K). PCA plots showed that the results within each group were relatively homogeneous and stable (S2B). Volcano plots revealed that a large number of genes were significantly up- or down-regulated upon IL-1 β intervention and YQYXQF treatment (Fig. 3L, S2C). GO analysis indicated that YQYXQF treatment mainly affected biological processes such as oxidative stress and the regulation of MAPK-related molecules (Fig. 3M, S2D). KEGG pathway enrichment analysis demonstrated that the candidate targets were significantly enriched in several classical signaling pathways, including the MAPK signaling pathway, TNF signaling pathway, and PI3K-Akt signaling pathway (Fig. 3N, S2E). In addition, analysis of PRKCA expression showed that PRKCA was significantly increased in the model group, whereas YQYXQF intervention suppressed this upregulation (Fig. 3O, S2F). GSEA enrichment analysis also revealed significant enrichment of the MAPK signaling pathway and ferroptosis-related indicators (Fig. 3P–Q, S2G–H).

These results indicate that the PRKCA-MAPK/ERK signaling axis may serve as the main mechanistic pathway underlying the therapeutic effects of YQYXQF on KOA.

Integrated analysis of potential targets of YQYXQF in the treatment of KOA using network pharmacology

First, a total of 77 major active constituents of YQYXQF were identified by LC-MS/MS, of which 38 were detected in positive ion mode and 39 in negative ion

mode (Fig. 4A, Table S1). Subsequently, the components absorbed into the serum following YQYXQF administration were analysed. After background subtraction by comparison with blank serum, 25 major active components were identified in the serum, including Daidzin, Umbelliferone, Scopolin, and Isoastragaloside I, among others (S3A, Table S3).

To identify potential therapeutic targets of YQYXQF for the treatment of KOA and to validate the transcriptomic findings, a network pharmacology analysis was performed (Fig. 4B). A Venn diagram revealed 298 overlapping targets between YQYXQF and KOA, which were considered as potential therapeutic targets of YQYXQF (Fig. 4C). The network diagram of key protein nodes showed that MAPK and PRKCA exhibited high connectivity, suggesting their central roles in the signal transduction and regulatory networks (Fig. 4D). GO enrichment analysis indicated that the candidate targets were primarily enriched in biological processes such as the “stress-activated MAPK cascade”, “regulation of mitochondrial membrane potential”, and “response to oxidative stress” (S3B). KEGG pathway enrichment analysis revealed significant enrichment of the candidate targets in several classical signalling pathways, including the MAPK, TNF, and NF- κ B signalling pathways (S3C). Based on these findings, we subsequently focused on validating the involvement of PRKCA in the therapeutic mechanism of YQYXQF against KOA. Molecular docking results for PRKCA revealed that several compounds, including Scopolin, Divaricataester B, and Daidzin, were capable of binding to the active pocket of the PRKCA protein through hydrophobic interactions and multiple hydrogen bonds (S3D). Subsequently,

molecular dynamics simulations were performed focusing on the three compounds mentioned above. The simulation trajectories indicated that all three compounds exhibited high conformational stability (Fig. 4E–H, S3E). Further analysis of key interactions for the three complexes revealed that the number of hydrogen bonds and hydrophobic contacts persisted throughout the simulation (Fig. 4I–J). MM/GBSA analysis demonstrated that the total binding free energies for all three compounds were negative, indicating spontaneous binding to PRKCA, with van der Waals (VDWAALS) and electrostatic (EEL) interactions serving as the primary driving forces (Fig. 4K). CETSA was performed to validate the molecular docking predictions. The results showed a rightward shift of the Tm50 following YQYXQF intervention, suggesting that the relevant active components could directly bind to PRKCA(S3F). Taken together, these findings support the identification of PRKCA as a target for intervening in KOA progression, and suggest that YQYXQF may exert its therapeutic effects in KOA by influencing PRKCA expression.

Knockdown of PRKCA Suppresses ERK Phosphorylation and Alleviates Mitochondrial Oxidative Stress and Ferroptosis In Vitro

To investigate how PRKCA influences knee osteoarthritis, siRNA was used to knock down PRKCA expression in vitro (Fig. 5A). RT-qPCR and Western blot analyses confirmed that transfection with siRNA successfully knocked down PRKCA in mouse primary chondrocytes (Fig. 5B–C, S4A). Building on the previous findings from single-cell and transcriptomic analyses suggesting that the activation of the MAPK signaling pathway in chondrocytes during KOA is closely linked to PRKCA,

we next investigated whether PRKCA influences the phosphorylation of ERK, JNK, and p38 in mouse primary chondrocytes. Western blot results demonstrated that IL-1 β induction significantly increased the phosphorylation levels of all three kinases. However, upon knockdown of PRKCA, the phosphorylation level of ERK was reduced, whereas no significant decreasing trend was observed for JNK and p38. This indicates that in chondrocytes, PRKCA primarily exerts its effects by promoting ERK phosphorylation (Fig. 5D–E). Western blot analysis of the mitochondrial oxidative stress marker SOD2 and cartilage matrix-related proteins revealed that knockdown of PRKCA significantly upregulated SOD2 protein expression and markedly improved the cartilage matrix (Fig. 5F–G). Confocal imaging of GPX4 and COL2 showed a significant increase in fluorescence intensity for both proteins following PRKCA knockdown, indicating attenuated ferroptosis and restored matrix integrity (Fig. 5H–I). RT-qPCR results further confirmed that PRKCA knockdown alleviated mitochondrial oxidative stress and ferroptosis while promoting cartilage matrix repair in vitro (Fig. 5J). Taken together, these observations preliminarily suggest that PRKCA may exacerbate mitochondrial oxidative stress and ferroptosis in chondrocytes under inflammatory conditions by promoting activation of the MAPK/ERK signaling pathway.

Activation of ERK phosphorylation attenuates the protective effects of PRKCA knockdown on chondrocytes

To further elucidate the regulatory role of PRKCA in knee osteoarthritis, validation was performed using the ERK-specific phosphorylation agonist Ceramide

C6 [18,19]. Western blot analysis of ERK demonstrated that the Ceramide C6 treatment successfully enhanced the level of ERK phosphorylation (Fig. 6F–G). Detection of mitochondrial membrane potential, ROS, and ATP levels revealed that specific knockdown of PRKCA using small interfering RNA significantly improved mitochondrial membrane potential, reduced ROS expression, and restored ATP levels in chondrocytes compared with the IL-1 β -treated group. In contrast, pre-treatment with Ceramide C6 led to a decrease in mitochondrial membrane potential, an increase in ROS expression, and a reduction in ATP levels (Fig. 6A–D, S4D). The results of MDA and BDPY C11 assays showed that Ceramide C6 intervention significantly elevated lipid peroxidation levels (Fig. 6E, S4B–C). Furthermore, Western blot results indicated that activation of ERK phosphorylation significantly downregulated SOD2 protein expression, enhanced mitochondrial oxidative stress, and worsened the cartilage matrix (Fig. 6F–G). RT-qPCR and immunofluorescence results further confirmed that the effects of PRKCA on chondrocyte ferroptosis and matrix anabolism/catabolism are dependent on the MAPK/ERK pathway (Fig. 6H–J, S4E). These experimental findings strongly demonstrate the feasibility of treating KOA by targeting the PRKCA–MAPK/ERK signalling axis.

YQYXQF improves chondrocyte matrix via regulating the PRKCA–MAPK/ERK signalling axis

The expression levels of PRKCA and p-ERK were examined using confocal microscopy. Compared with the model group, treatment with YQYXQF significantly reduced the expression of both PRKCA and p-ERK (Fig. 7A–C). In addition,

RT-qPCR results demonstrated a decrease in PRKCA expression at the gene level (Fig. 7D). Western blot analysis of PRKCA and ERK further indicated that YQYXQF is capable of suppressing the progression of osteoarthritis by modulating the PRKCA–MAPK/ERK signalling axis (Fig. 7E, S5A-B). Furthermore, we further validated the above observations in vivo. H&E images showed no obvious adverse effects of YQYXQF on the major organs (S5C). IHC results showed that the expression of SOD2 (a marker of inhibited mitochondrial oxidative stress), GPX4 (a marker of suppressed ferroptosis), and COL II (a marker of the anabolic metabolism of the ECM) was significantly upregulated following YQYXQF treatment. In parallel, the expression of PRKCA and MMP13 (a marker of the catabolic metabolism of the ECM) was markedly downregulated. Additionally, IF analysis was performed to assess the inflammatory status in the knee joints of each group. The inflammatory status in the knee joints was assessed by IF. This demonstrated increased IL-1 β expression in the model group relative to the control group, which was subsequently lowered by YQYXQF treatment (Fig. 7F–G). Additionally, we performed rescue experiments using the PRKCA agonist PMA. Both RT-qPCR and Western blot analyses demonstrated that YQYXQF exerts its therapeutic effect on KOA through PRKCA(S5D-G). Integrating the findings from both in vivo and in vitro experiments, YQYXQF alleviates the progression of osteoarthritis by regulating the PRKCA–MAPK/ERK signalling axis. This regulation inhibits mitochondrial oxidative stress and ferroptosis in chondrocytes, promotes the synthesis of the cartilage matrix, and suppresses its catabolism.

Discussion

KOA, as a prominent degenerative joint disorder globally, has consistently drawn significant research attention regarding its epidemiological characteristics and pathological mechanisms. While numerous therapeutic strategies for KOA exist today, each presents its own limitations [20,21]. In recent years, with the deepening of research in traditional Chinese medicine, its therapeutic principles of holistic regulation, treatment based on syndrome differentiation, and simultaneous attention to both root causes and clinical presentations have demonstrated distinct advantages in the prevention and management of KOA, gaining increasing clinical application [22]. YQYXQF, a traditional Chinese herbal formula originating from Zhejiang, China, aligns with this therapeutic principle. It has long been used clinically for treating KOA with favourable therapeutic outcomes. However, the lack of mechanistic research on this formula has limited its further development. Through integrated in vivo and in vitro experiments, combined with multi-omics and network pharmacology, this study systematically elucidated that PRKCA is aberrantly highly expressed in the context of KOA. Furthermore, our study confirms that YQYXQF treats KOA by regulating the PRKCA–MAPK/ERK signalling axis to inhibit mitochondrial oxidative stress and alleviate ferroptosis. Thereby, we establish an evidence chain for YQYXQF linking its "herbal components – acting targets and downstream pathways – cartilage protective effects"(Fig. 8).

PRKCA, a key member of the protein kinase C family, plays a significant regulatory role in various cellular biological processes, including ferroptosis [23].

Existing research suggests that PRKCA expression is significantly elevated in osteoarthritis, supporting its candidacy as a therapeutic target for KOA [24,25]. Previous studies in oncology have established that PRKCA mediates ferroptosis in prostate cancer cells by regulating GPX4 expression [26]. In the field of neuroscience, inhibiting PRKCA can reverse the effects of silenced AEBP1 expression on neuronal ROS production and ferroptosis [27]. This study, through single-cell analysis of public databases from knee osteoarthritis patients and transcriptomic analysis of mouse primary chondrocytes, confirmed that PRKCA expression is significantly elevated in both chondrocytes from human OA patients and mouse primary chondrocytes under an IL-1 β -simulated osteoarthritic environment. Furthermore, knockdown of PRKCA using siRNA in vitro significantly restored mitochondrial function and improved the cartilage matrix in chondrocytes. Collectively, these findings indicate that PRKCA is a potential therapeutic target for KOA.

The MAPK signalling pathway is recognised as playing a pivotal role in KOA progression and is closely associated with PRKCA, a key signalling molecule. Previous research has demonstrated that this pathway critically regulates inflammatory responses and matrix degradation, processes which contribute to the exacerbation of joint damage in osteoarthritis [28,29]. ERK, one of the three main branches of the MAPK signaling pathway, is closely associated with oxidative stress responses [30–32]. On one hand, factors such as inflammatory cytokine infiltration and mechanical stress within the OA microenvironment can activate ERK phosphorylation [33]. On the other hand, activated ERK further exacerbates OA

680 progression through pathways including promoting oxidative stress, suppressing
681 antioxidant defenses, and inducing matrix catabolism [34]. Therefore, inhibiting the
682 MAPK signaling pathway, particularly the ERK branch, represents a significant
683 research direction in the treatment of osteoarthritis. In this study, integrated analysis
684 of single-cell and transcriptomic data revealed the MAPK/ERK signaling pathway to
685 be a significantly enriched pathway in KOA. Notably, the results of Western blot
686 analysis for the three main branches of the MAPK signalling pathway in this study
687 showed that, under simulated KOA conditions, the effect of PRKCA on the MAPK
688 signalling pathway was primarily on ERK, whereas no significant effect was observed
689 on the phosphorylation levels of JNK or p38. These experimental findings are
690 consistent with previous studies, and may be attributed to the unique intracellular
691 environment of chondrocytes, as well as the relatively weak regulatory effect of
692 PRKCA on upstream mediators of the JNK and p38 pathways [35,36]. Building on
693 earlier findings, research suggests that PRKCA may activate MEK/ERK
694 independently of Raf and Ras [37]. To further elucidate the relationship
695 between PRKCA and the MAPK/ERK signaling pathway as well as mitochondrial
696 oxidative stress and ferroptosis, we employed siRNA to knock
697 down PRKCA expression and validated the findings using the ERK-specific
698 phosphorylation agonist Ceramide C6. The results demonstrated that in chondrocytes
699 under inflammatory conditions, knockdown of PRKCA effectively inhibited ERK
700 phosphorylation, improved mitochondrial function, alleviated ferroptosis, and thereby
701 restored the chondrocyte extracellular matrix. Conversely, intervention with the

ERK-specific phosphorylation agonist Ceramide C6 attenuated the protective effects conferred by PRKCA knockdown in chondrocytes, exacerbating mitochondrial oxidative stress, promoting ferroptosis, and enhancing abnormal degradation of the cartilage matrix. These results strongly indicate that the influence of PRKCA on the progression of KOA is mediated primarily through the MAPK/ERK signalling pathway.

Activation of ERK phosphorylation leads to mitochondrial oxidative stress, which exhibits a close causal relationship with ferroptosis [38]. Previous studies have demonstrated that the inflammatory microenvironment associated with KOA progression triggers the generation of substantial amounts of free radicals and ROS within chondrocytes, with mitochondria being a primary target of such attacks [39]. Mitochondria with impaired function further produce excessive reactive oxygen species while their intrinsic antioxidant capacity declines, resulting in mitochondrial oxidative stress and establishing a vicious cycle [40]. Excessive ROS attack polyunsaturated fatty acids, initiating a lipid peroxidation chain reaction that ultimately leads to ferroptosis [41,42]. In this study, we assessed mitochondrial functional status by measuring mitochondrial membrane potential, intracellular ROS levels, and the expression of the mitochondrial oxidative stress marker SOD2. Furthermore, fatty acid metabolism abnormalities were evaluated using MDA, while the extent of ferroptosis was determined based on GPX4 expression [43,44]. Our study demonstrates that under IL-1 β -simulated KOA conditions in vitro, chondrocytes exhibited significantly impaired mitochondrial membrane potential, abnormally

elevated levels of ROS and MDA, and concomitant downregulation of SOD2 and GPX4 expression. Notably, knockdown of PRKCA markedly ameliorated these pathological alterations and restored the cartilage matrix. Conversely, Ceramide C6 intervention attenuated the therapeutic effect of PRKCA knockdown on mitochondrial function, leading to a rebound in the extent of ferroptosis. In summary, this study demonstrates the pivotal role of modulating the PRKCA-MAPK/ERK signalling axis in inhibiting mitochondrial oxidative stress in chondrocytes, alleviating ferroptosis, and ultimately delaying the progression of KOA.

TCM compound formulations have long been employed in the management of KOA, characterised by their multi-component nature, systematic therapeutic approach, and low side-effect profile [22,45]. In previous studies, substantial evidence has confirmed that TCM compound formulations demonstrate considerable efficacy in improving mitochondrial function for the treatment of KOA. Research by Jie et al. (2025) demonstrated that Quzhi Tang could alleviate oxidative stress and restore mitochondrial function by regulating the GPNMB/Nrf2/NF- κ B signalling pathway [46]. Meanwhile, Yang et al. (2024) confirmed that the Gubi Zhitong formula could mitigate OA-related inflammation and cartilage damage by modulating BNIP3L-mediated mitophagy [47]. In this study, primary mouse chondrocytes were treated with YQYXQF-containing serum. Comparative analysis between the drug-containing serum intervention group and the model group revealed that YQYXQF significantly improved mitochondrial morphology as observed by transmission electron microscopy, enhanced ATP production, and restored

mitochondrial membrane potential as well as the expression levels of SOD2 and GPX4. Furthermore, YQYXQF intervention suppressed the IL-1 β -induced elevation of ROS and the disturbance in fatty acid metabolism in chondrocytes. In addition, the in vivo experiments in the DMM-induced KOA mouse model, along with the rescue experiments using Erastin, further validated the above findings. Notably, transmission electron microscopy revealed that some mitochondria in the model group exhibited swelling and rounding. This morphological change differs from the typical ferroptosis-associated phenotype, which is characterised by mitochondrial shrinkage and increased membrane density. The observed swelling may be attributed to IL-1 β stimulation, which induces pathological opening of certain channels in the mitochondrial membrane, leading to an imbalance in intra- and extra-mitochondrial osmotic pressure [48]. Integrating data from both in vivo and in vitro experiments, this study demonstrates that YQYXQF exerts its therapeutic effect on KOA by inhibiting mitochondrial oxidative stress in chondrocytes and alleviating ferroptosis.

Molecular docking and molecular dynamics simulations serve as a critical bridge connecting traditional Chinese medicine and modern medical science, playing a pivotal role in elucidating the mechanisms of action of herbal constituents at their target sites. This study performed network pharmacological validation and molecular docking based on the major compounds identified in YQYXQF via LC-MS/MS analysis. According to LC-MS/MS analysis, YQYXQF comprises multiple bioactive compounds such as flavonoids, saponins, polysaccharides, and terpenoids. Among these, approximately 25 major active constituents were absorbed into the serum. The

majority of these compounds have been previously confirmed in other studies to be effective in treating diseases related to oxidative stress and ferroptosis. Scopolin, a coumarin derived from *Saposhnikovia divaricata*, can inhibit oleic acid-induced lipid accumulation in HepG2 cells, an effect mediated through the upregulation of SIRT1 activity [49]. Other studies also indicate it can alleviate osteoarthritis in rats by suppressing inflammation [50]. Similarly, the isoflavone glycoside Daidzin, sourced from *Astragalus membranaceus*, mitigates osteoarthritis progression by modulating FoxO1 expression to inhibit endoplasmic reticulum stress [51]. In RAW264.7 cells, Daidzin attenuated the LPS-induced inflammatory response via suppression of the MAPK signaling pathway [52]. Following molecular docking, the top three YQYXQF active compounds with the lowest binding energies—Scopolin, Daidzin, and Divaricataester B—were selected for molecular dynamics simulations. The results showed that all three active compounds spontaneously formed stable conformations with PRKCA through a series of intermolecular forces. More importantly, the subsequent CETSA results validated the molecular docking findings. In addition, immunohistochemistry of pathological sections, together with a series of experiments using the PRKCA agonist PMA, further confirmed the critical role of the PRKCA–MAPK/ERK signalling axis in the therapeutic effect of YQYXQF against KOA. Relevant studies have demonstrated that phosphorylation-mediated activation of ERK can directly regulate the occurrence of ferritinophagy, thereby inducing ferroptosis and disrupting oxidative homeostasis [53,54]. In addition, ERK can also modulate transcription factors such as NRF2,

790 FOXO, and CREB, thereby exacerbating oxidative stress and indirectly promoting
791 ferroptosis progression [24,55]. Although it is well established that ERK influences
792 oxidative stress and ferroptosis, we acknowledge that the present study did not
793 directly investigate how YQYXQF alleviates ferroptosis following the reduction of
794 ERK phosphorylation, and we plan to explore this aspect in greater depth in future
795 studies. In summary, this study is the first to systematically establish a complete
796 mechanistic chain in the field of KOA research, demonstrating that the PRKCA–
797 MAPK/ERK signalling axis regulates KOA progression by influencing mitochondrial
798 oxidative stress and ferroptosis in chondrocytes. Moreover, our findings elucidate that
799 YQYXQF exerts its therapeutic effect on KOA by directly targeting the PRKCA–
800 MAPK/ERK signalling axis, thereby inhibiting mitochondrial oxidative stress in
801 chondrocytes and alleviating ferroptosis.

802 Although the results of this study demonstrate that YQYXQF exerts a significant
803 therapeutic effect on KOA, certain limitations remain. As a traditional Chinese
804 medicine compound formulation, the therapeutic action of YQYXQF likely involves a
805 synergistic regulatory network comprising multiple components, multiple targets, and
806 multiple pathways. This study primarily focused on the PRKCA–MAPK/ERK
807 signalling axis and preliminarily confirmed its key role in the chondroprotective
808 effects of YQYXQF, as well as in the inhibition of mitochondrial oxidative stress and
809 alleviation of ferroptosis. However, this axis represents an important but not exclusive
810 mechanism underlying the therapeutic effects of YQYXQF; other potential signalling
811 pathways, synergistic interactions among active components, and crosstalk networks

among distinct targets remain to be fully elucidated. Furthermore, pharmacokinetic studies on YQYXQF remain at a relatively early stage, and further investigation is needed to facilitate its clinical translation in the future. Therefore, a more comprehensive understanding of the mechanism of action of YQYXQF in the treatment of KOA warrants further investigation, and we aim to explore its complex multi-target regulatory network and pharmacokinetic characteristics in greater depth in future studies.

Conclusion

In summary, this study validates the potential of targeting the PRKCA–MAPK/ERK signalling axis to suppress mitochondrial oxidative stress, alleviate ferroptosis, and thereby mitigate the progression of osteoarthritis. Furthermore, employing an integrated approach of network pharmacology, molecular docking, and molecular dynamics simulations, PRKCA was established as a pivotal target for YQYXQF in KOA treatment. These findings provide novel insights into the therapeutic mechanisms for KOA and open new avenues for treating this condition with Traditional Chinese Medicine.

Declarations

Ethics approval and consent to participate

Approval for the animal experiments conducted in this study was granted by the Animal Ethics Committee of Zhejiang Chinese Medical University (June 2025) (Approval No.: 20250623-16).

Consent for publication

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Mingchao Yuan: Writing – original draft, Data curation, Software, Investigation, Methodology. **Zhenglin Mei:** Writing – original draft, Investigation, Methodology; **Yanli Pan:** Investigation; Data curation, Software. **Weihui Qi:** Investigation, Data curation. **Duodan Zhang:** Investigation. **Liangping Zhang:** Funding acquisition, Investigation, Software. **Jingtao Hu:** Software, Visualization. **Wei Cheng:** Investigation. **Hanting Xia:** Writing – review & editing; **Hongting Jin:** Supervision, Funding acquisition, Writing – review & editing. **Hao Pan:** Supervision, Funding acquisition, Writing – review & editing. **Dong Wang:** Supervision, Project

Administration, Funding acquisition, Writing – review & editing.

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Figure legends:

Figure 1. Therapeutic effects of YQYXQF in a mouse DMM model. (A) Schematic diagram of the in vivo experimental design. (B–C) Open-field test in mice ($n = 5$). (D–E) Gait analysis in mice ($n = 5$). (F–I) Representative micro-CT images of mouse knee joints and quantitative analysis of Tb.Sp, Tb.Th and BV/TV. (J–K) Representative H&E stained images of mouse knee joints and OARSI scoring ($n = 5$). (L) Representative images of mouse knee joints stained with SO-FG. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, All data represent mean $\pm$ SD).

Figure 2. YQYXQF protects the chondrocyte extracellular matrix by inhibiting mitochondrial oxidative stress and ferroptosis. (A) Representative transmission electron microscopy images of mitochondrial morphology in chondrocytes treated with YQYXQF-containing serum at different concentrations. (B–C) Representative JC-1 fluorescence images and quantitative analysis of primary mouse chondrocytes ($n = 3$). (D–E) Representative ROS fluorescence images and quantitative analysis of primary mouse chondrocytes ($n = 3$). (F) Quantitative analysis of MDA levels ($n = 5$). (G–H) Representative GPX4 fluorescence images and quantitative analysis ($n = 3$). (I–J) Representative Western blot bands and quantitative analysis of SOD2 and matrix-related protein expression levels ($n = 3$). (K) RT-qPCR results of related genes ($n = 6$). ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, All data represent mean $\pm$ SD).

Figure 3. Single-cell RNA-seq and Transcriptomics suggest PRKCA as a potential therapeutic target of YQYXQF. (A–B) Schematic workflow of the single-cell omics analysis using public datasets (OA: $n = 4$; Normal: $n = 3$) and quality control of the data. (C) t-SNE plot of knee joint tissue samples. (D) Volcano plot of chondrocyte clusters based on single-cell data. (E) GSEA analysis of the MAPK signalling pathway in chondrocyte clusters. (F) RT-qPCR results for PRKCA in primary mouse chondrocytes ($n = 6$). (G–H) Representative IF images and quantitative analysis of PRKCA in primary mouse chondrocytes ($n = 3$). (I–J) Quantitative analysis of IHC staining for PRKCA ($n = 5$). (K) Schematic workflow of the transcriptomic analysis of primary mouse chondrocytes ($n = 4$). (L) Volcano plot of differentially expressed genes in primary cells from the control and model groups. (M–N) GO and KEGG enrichment analyses. (O) Expression of PRKCA in primary mouse chondrocytes following IL-1 β intervention. (P) GSEA analysis of the MAPK signalling pathway. (Q) GSEA analysis of ferroptosis. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, All data represent mean $\pm$ SD).

Figure 4. Molecular docking and dynamics simulations of key active constituents of YQYXQF with PRKCA. (A) LC/MS analysis of YQYXQF and its major constituent compounds. (B) Schematic of the analytical workflow. (C) Venn diagram of potential therapeutic targets based on the overlap between KOA-related targets and those of the major active compounds in YQYXQF. (D) Network diagram of key protein nodes. (E) RMSD values over time and their density distribution for the complexes of PRKCA with Daidzin, Divaricataester B, and Scopolin. (F) Root mean

square fluctuation (RMSF) of protein residues. (G) Radius of gyration (Rg) of the protein over time and its density distribution. (H) Minimum distance between the protein and ligand over time and its density distribution. (I) Number of hydrogen bonds over time in the complexes of PRKCA with Daidzin, Divaricataester B, and Scopolin. (J) Number of hydrophobic interactions over time in the complexes of PRKCA with Daidzin, Divaricataester B, and Scopolin. (K) Energy component analysis of the complexes of PRKCA with Daidzin, Divaricataester B, and Scopolin.

Figure 5. Knockdown of PRKCA improves chondrocyte matrix homeostasis. (A) Schematic diagram of the experimental workflow. (B) Validation of si-PRKCA knockdown efficiency at the gene expression level ($n=6$). (C) Validation of si-PRKCA knockdown efficiency at the protein expression level ($n=3$). (D–E) Representative Western blot bands and quantitative analysis of the MAPK signalling pathway ($n=3$). (F–G) Representative Western blot bands and quantitative analysis of SOD2 and matrix-related protein expression levels ($n=3$). (H–I) Representative fluorescence images and quantitative analysis of GPX4 and COL2 ($n=3$). (J) RT-qPCR results of related genes ($n=6$). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, All data represent mean $\pm$ SD).

Figure 6. Activation of ERK phosphorylation attenuates the therapeutic effects of PRKCA knockdown on chondrocytes. (A–B) Representative JC-1 fluorescence images and quantitative analysis ($n=3$). (C–D) Representative fluorescence images and quantitative analysis of ROS expression ($n=3$). (E) Quantitative analysis of MDA ($n=5$). (F–G) Representative Western blot bands and quantitative analysis of ERK, SOD2, and matrix-related proteins ($n=3$). (H) RT-qPCR results of related genes ($n=6$). (I–J) Representative fluorescence images and quantitative analysis of GPX4 and matrix-related proteins ($n=3$). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, All data represent mean $\pm$ SD).

Figure 7. YQYXQF alleviates KOA progression via the PRKCA–MAPK/ERK signalling axis. (A–C) Representative fluorescence images and quantitative analysis of PRKCA and p-ERK ($n=3$). (D) RT-qPCR results for PRKCA ($n=6$). (E) Representative Western blot bands of PRKCA and ERK ($n=3$). (F–G) IHC and IF analyses for in vivo validation of the mechanism by which YQYXQF treats KOA ($n=5$). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, All data represent mean $\pm$ SD).

Figure 8. Schematic diagram of the molecular mechanism underlying the therapeutic effect of YQYXQF on KOA.

Figure S1. (A) Cell viability measured by the CCK-8 assay to determine the serum intervention concentration ($n=5$). (B–D) Flow cytometric detection of ROS expression in primary mouse chondrocytes ($n=3$). The percentage of ROS-positive cells and the median fluorescence intensity were quantified. (E) ATP levels measured using an ATP assay kit ($n=3$). (F–G) Representative fluorescence images of BDPY

C11 staining and quantitative analysis ($n=3$). (H–J) Representative fluorescence images of COL2 and MMP13 and quantitative analysis ($n=3$). (K) RT-qPCR results for ADAMTS5 and ACAN ($n=6$). (M) RT-qPCR results from the ferroptosis rescue experiment ($n=6$). ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

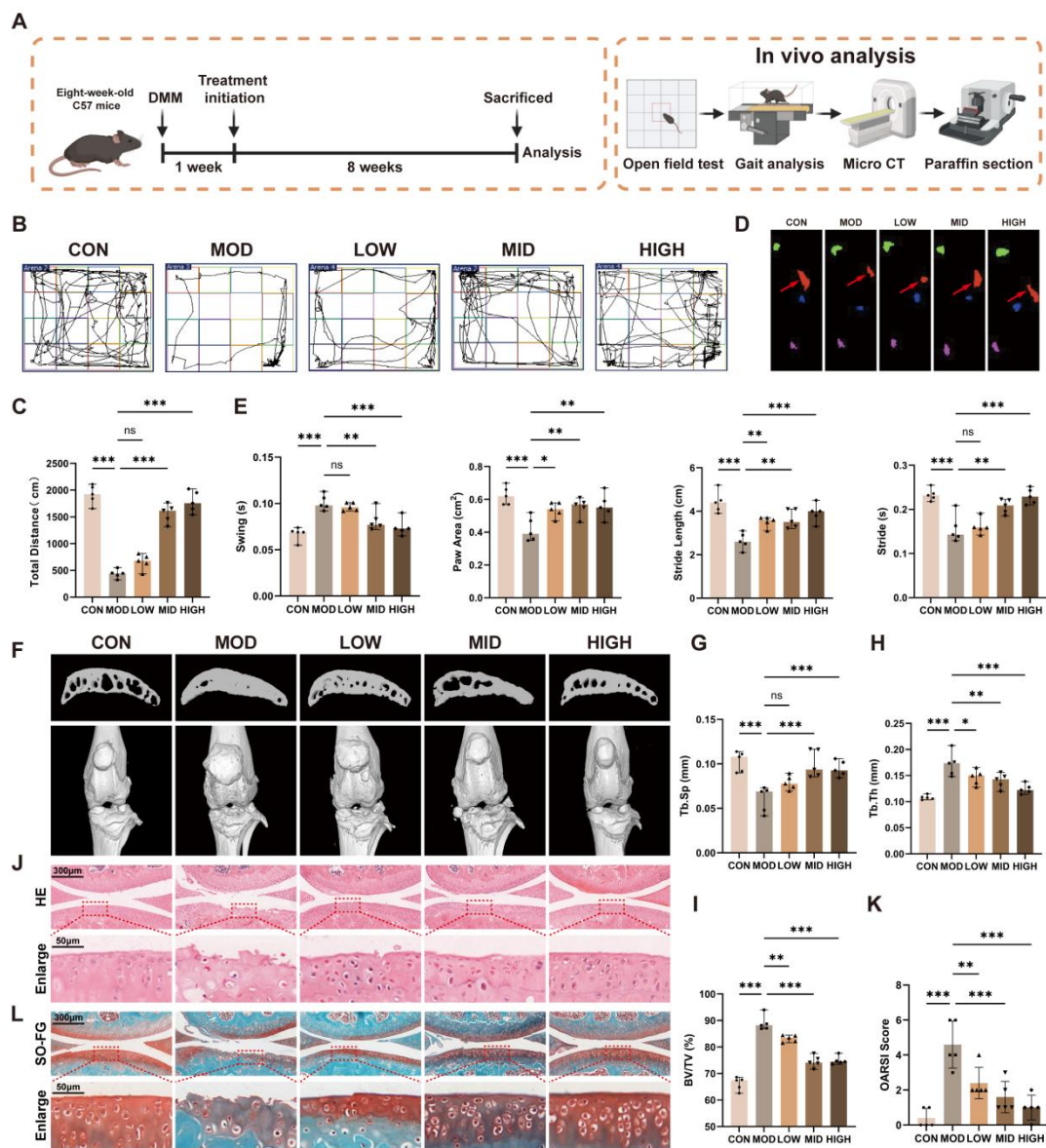
Figure S2. (A) Expression of PRKCA in each cell type based on single-cell data. (B) Principal component analysis (PCA) plot of the transcriptome sequencing data from primary mouse chondrocytes. (C) Volcano plot of differentially expressed genes between the treatment group and the model group in primary mouse chondrocytes. (D) GO functional enrichment analysis between the YQYXQF treatment group and the model group. (E) KEGG pathway enrichment analysis between the YQYXQF treatment group and the model group. (F) Expression of PRKCA. (G–H) GSEA analysis of the MAPK signalling pathway and ferroptosis influenced by YQYXQF. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Figure S3. (A) LC-MS/MS analysis of YQYXQF-containing serum. (B) GO functional enrichment analysis. (C) KEGG pathway enrichment analysis. (D) Molecular docking of selected active components of YQYXQF (Daidzin, Divaricataester B, and Scopolin). (E) B-factor plot of the complexes of PRKCA with Daidzin, Divaricataester B, and Scopolin. (F) CETSA analysis of YQYXQF binding to PRKCA.

Figure S4. (A) Representative Western blot bands validating the knockdown efficiency of si-PRKCA. (B–C) Representative BDPY C11 fluorescence images and quantitative analysis ($n=3$). (D) ATP level measurement ($n=3$). (E) Representative fluorescence image of MMP13. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

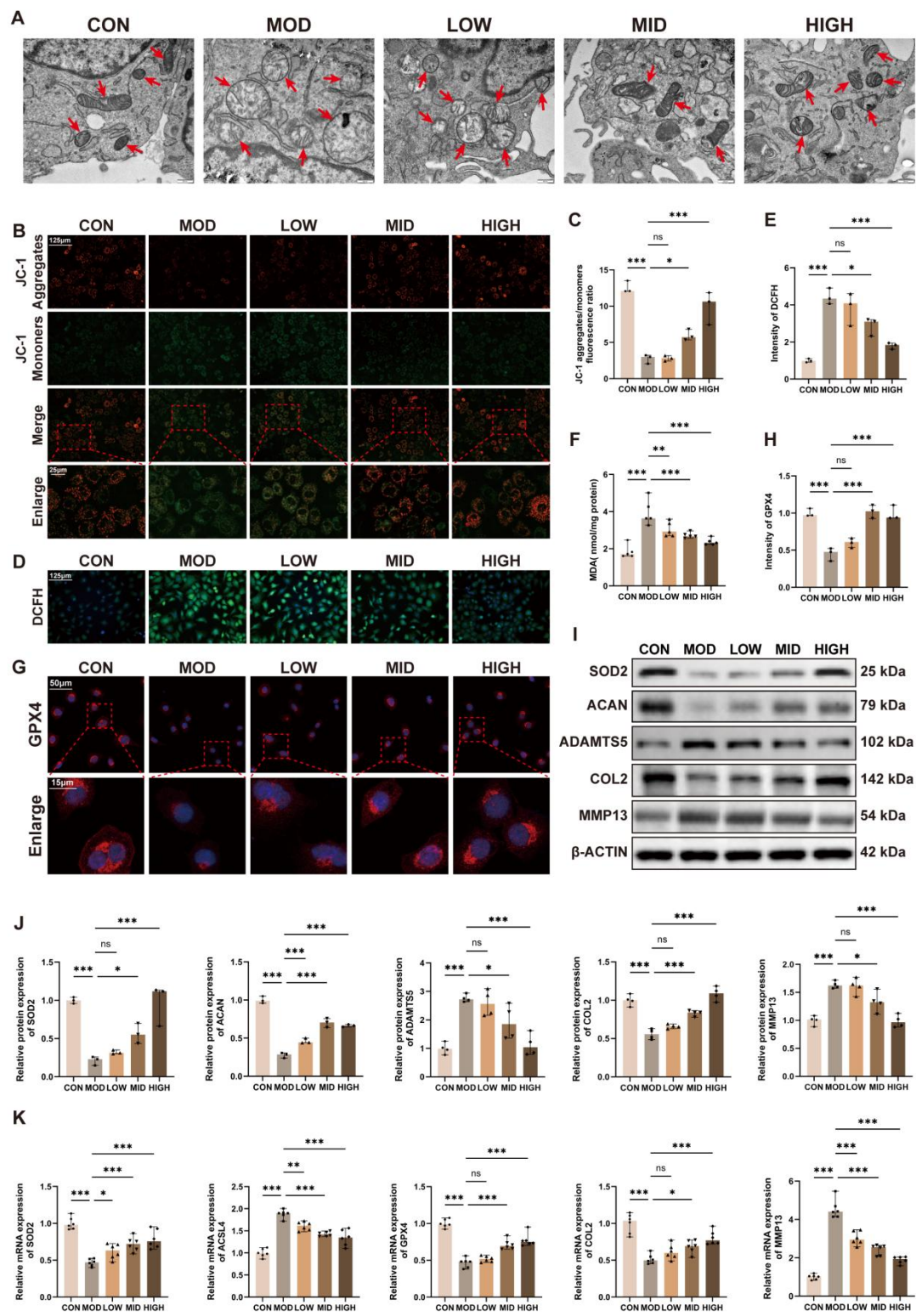
Figure S5. (A–B) Quantitative Western blot analysis of PRKCA and ERK ($n=3$). (C) Representative images of visceral H&E staining. (D–E) RT-qPCR results following PRKCA activation with PMA ($n=6$). (F–G) Western blot results following PRKCA activation with PMA ($n=3$). ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

1154 **Figures**
1155 **Figure 1**



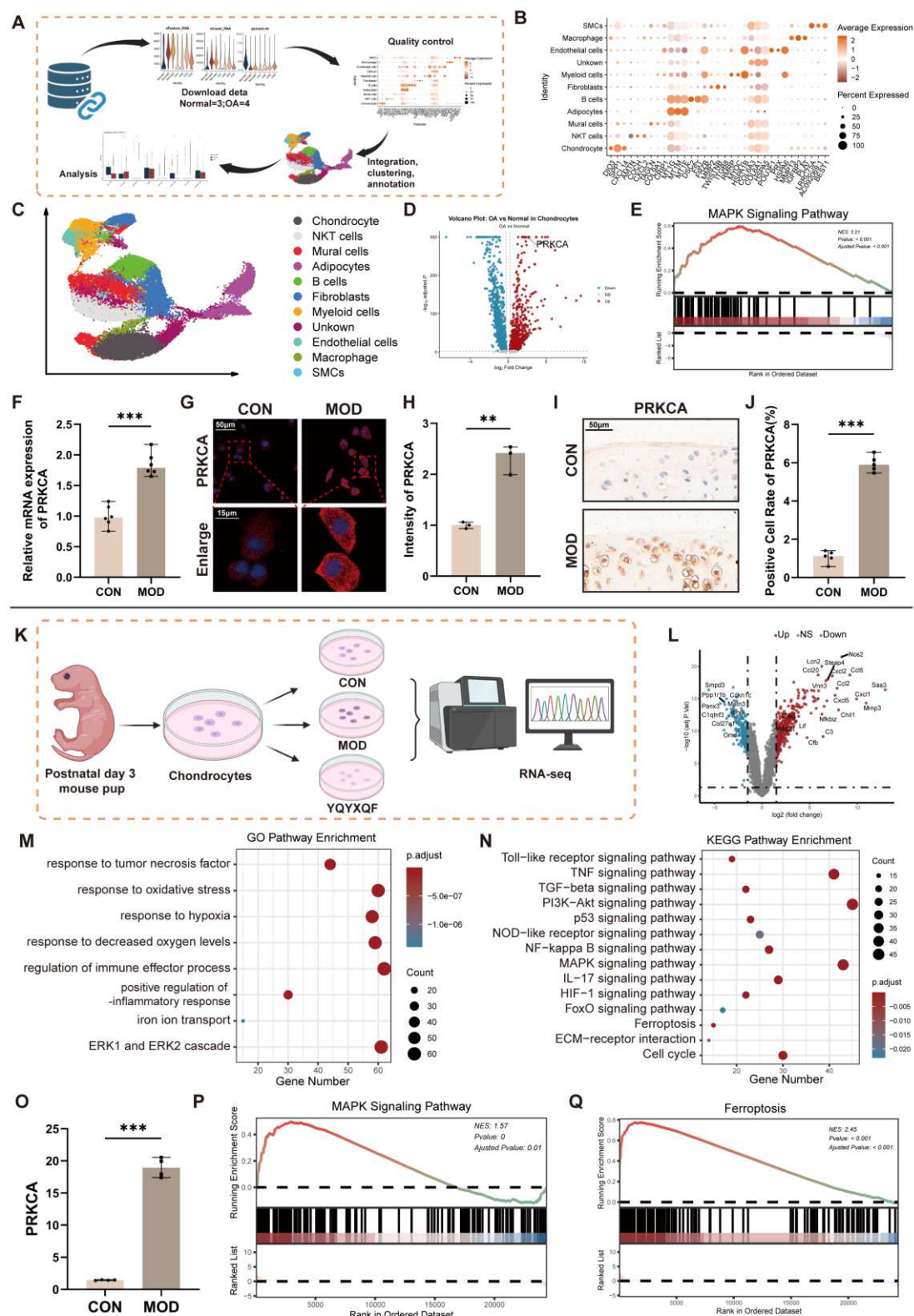
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1157 **Figure 2**

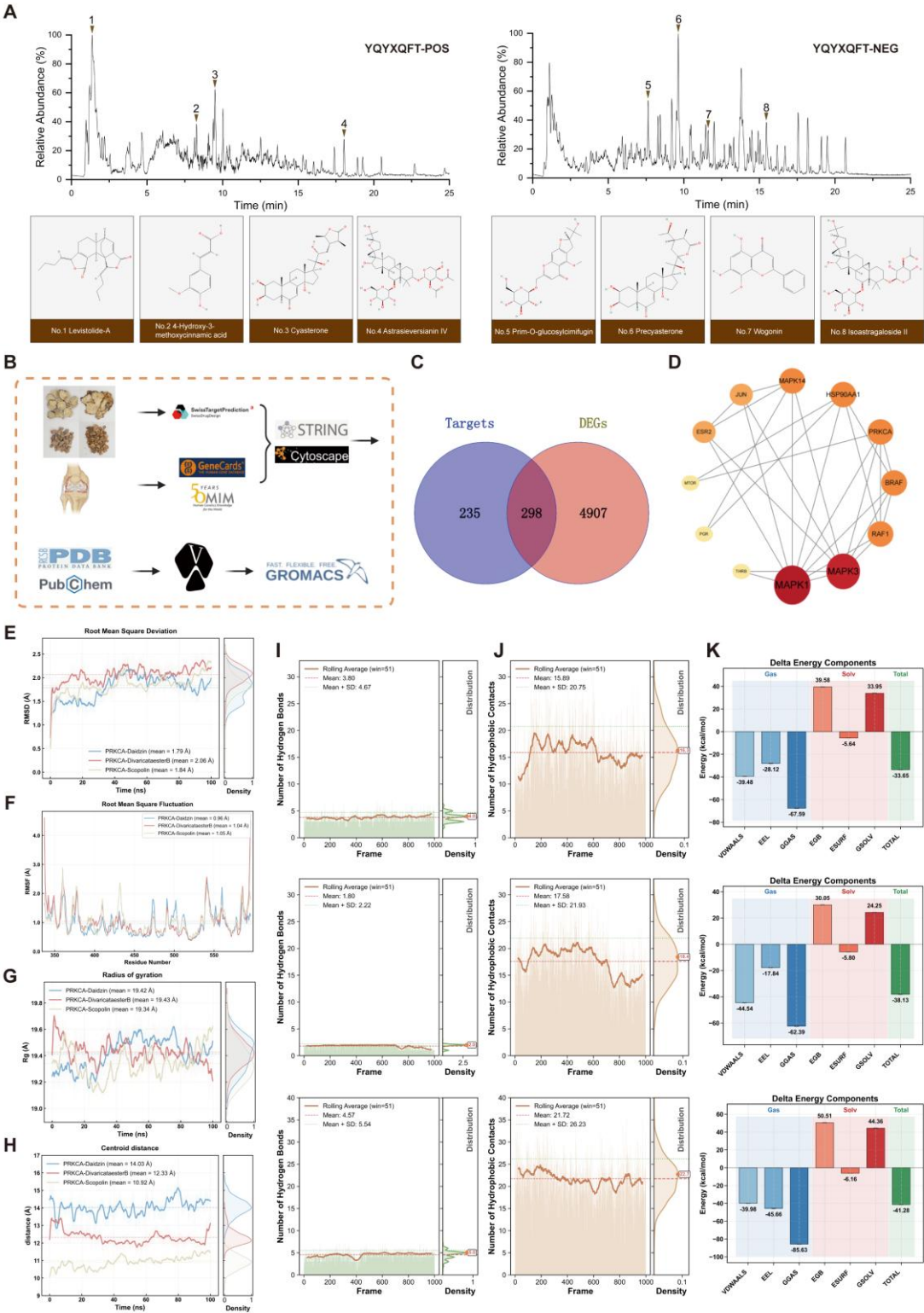


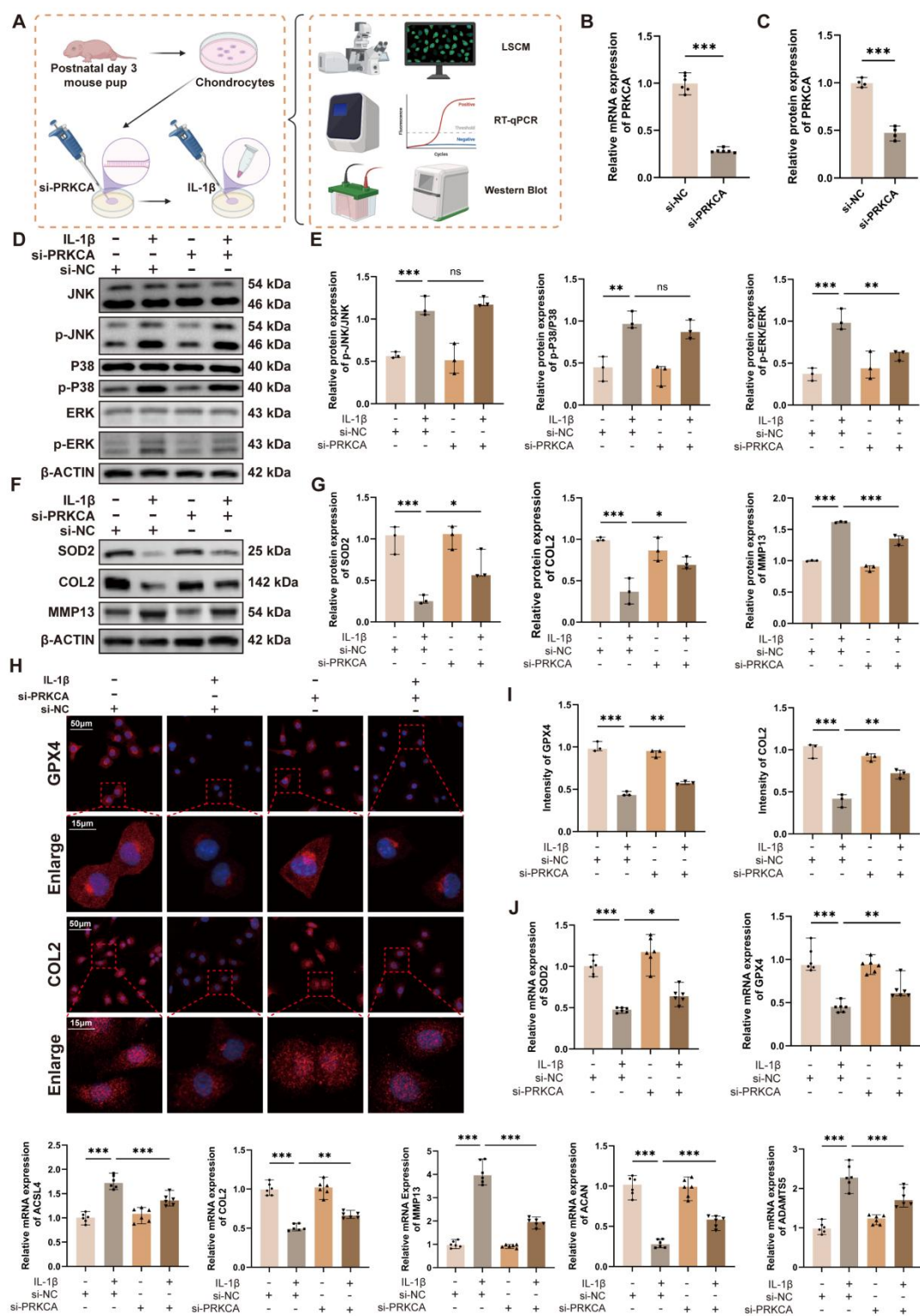
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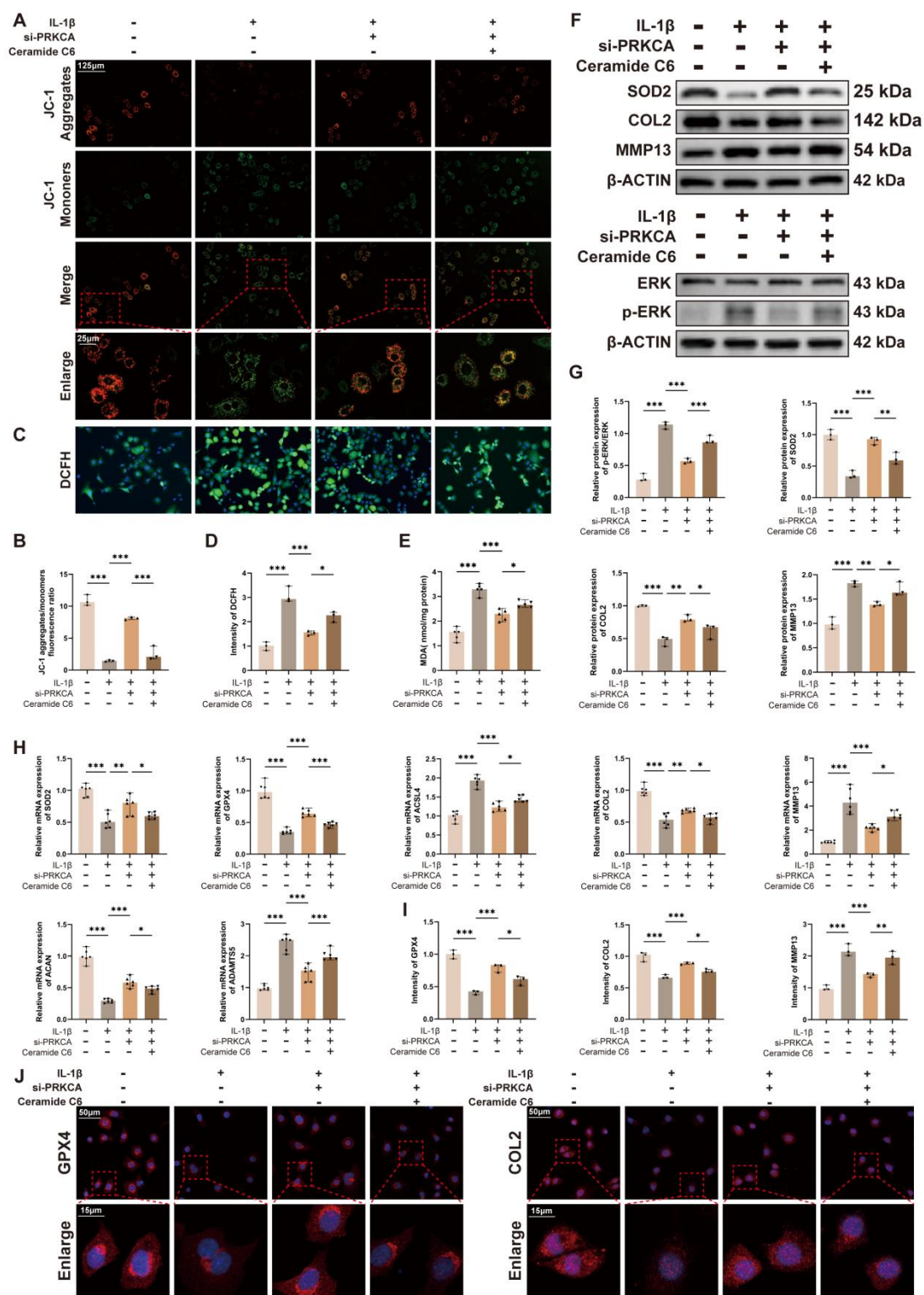
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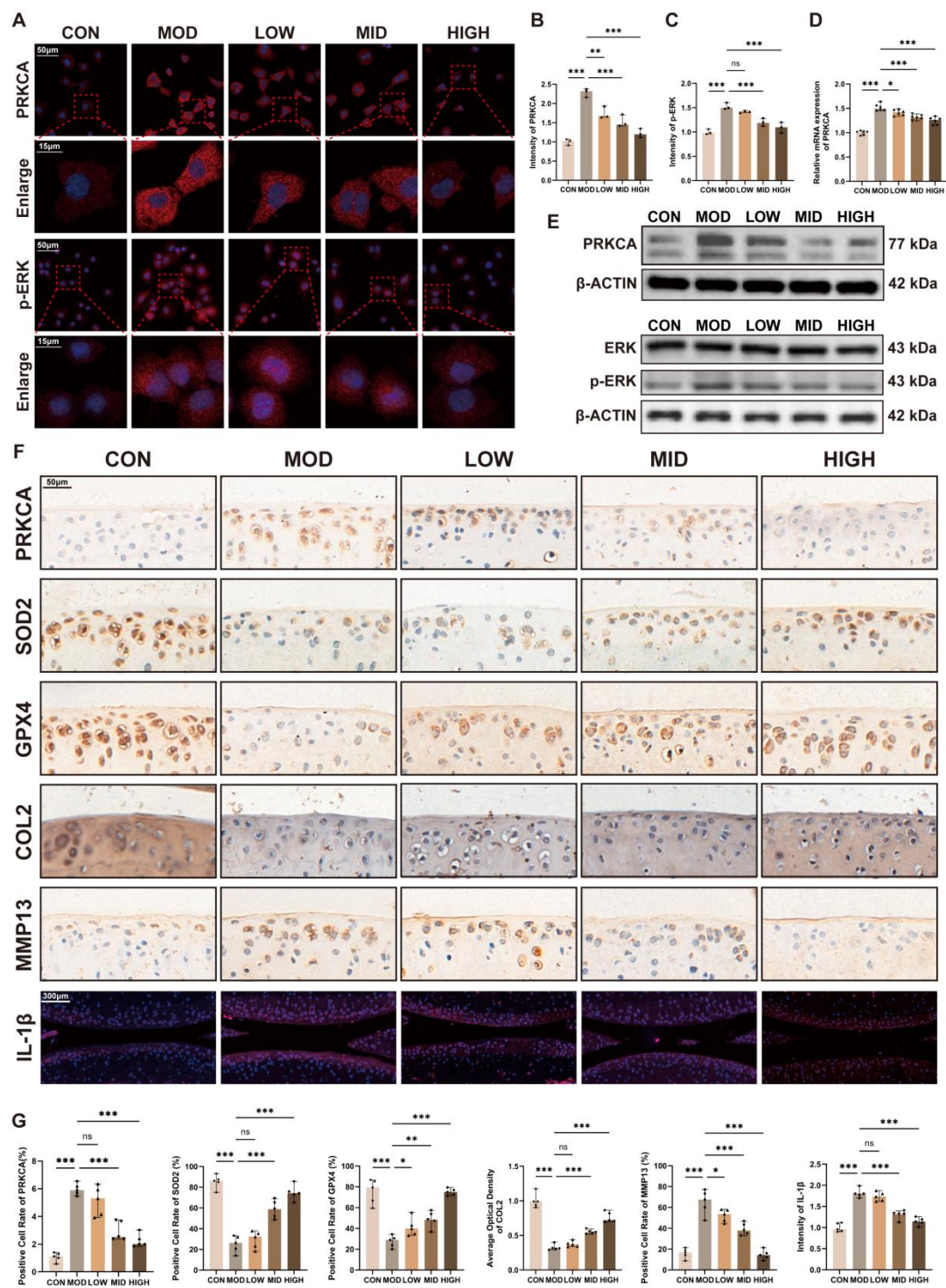


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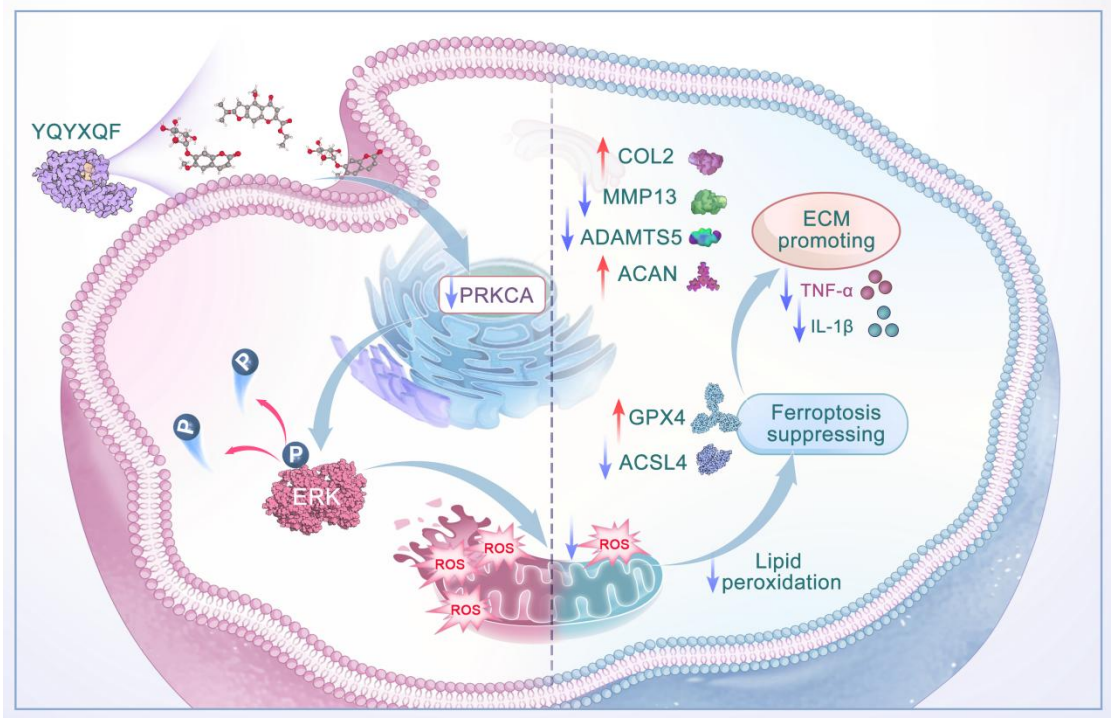






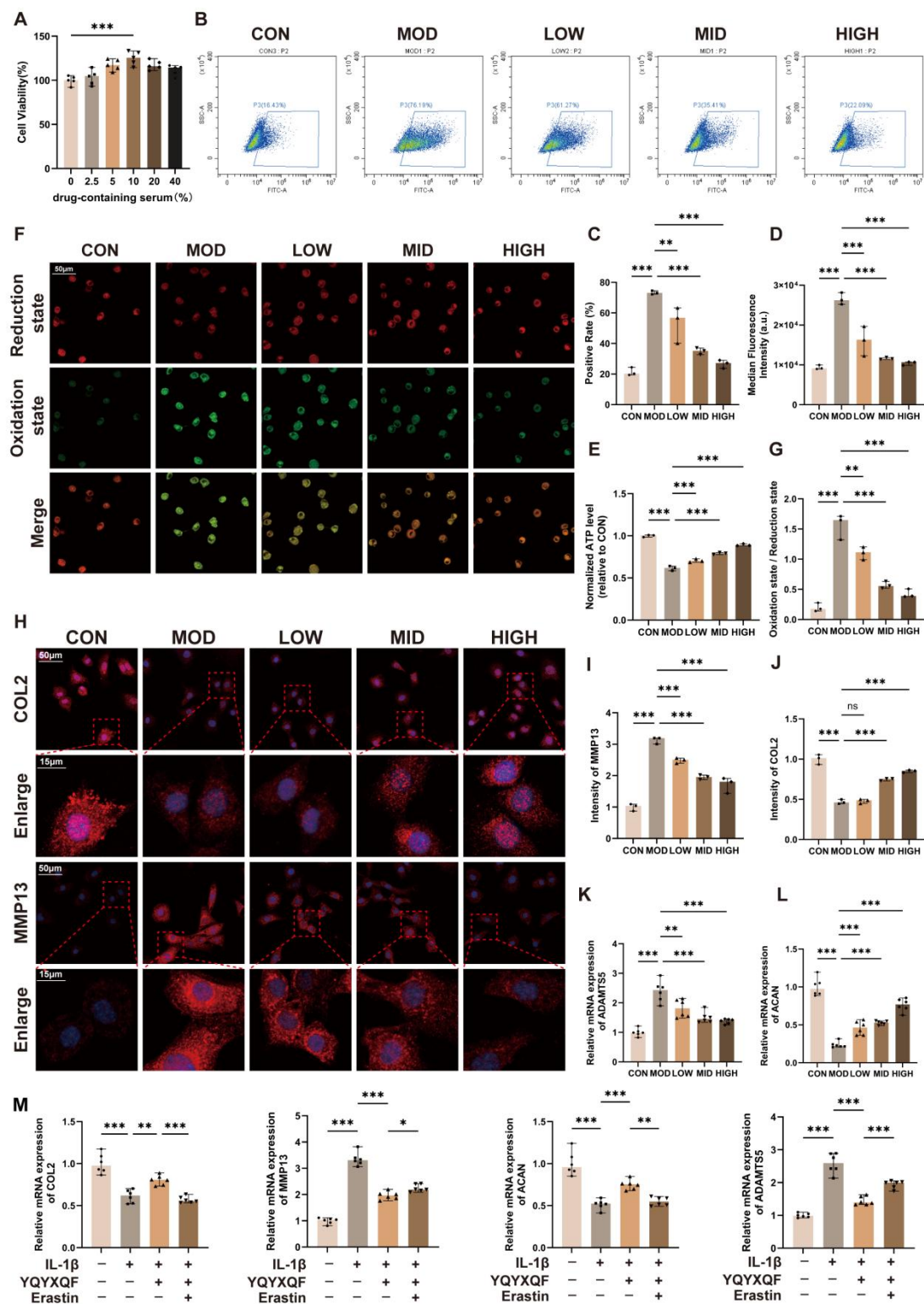


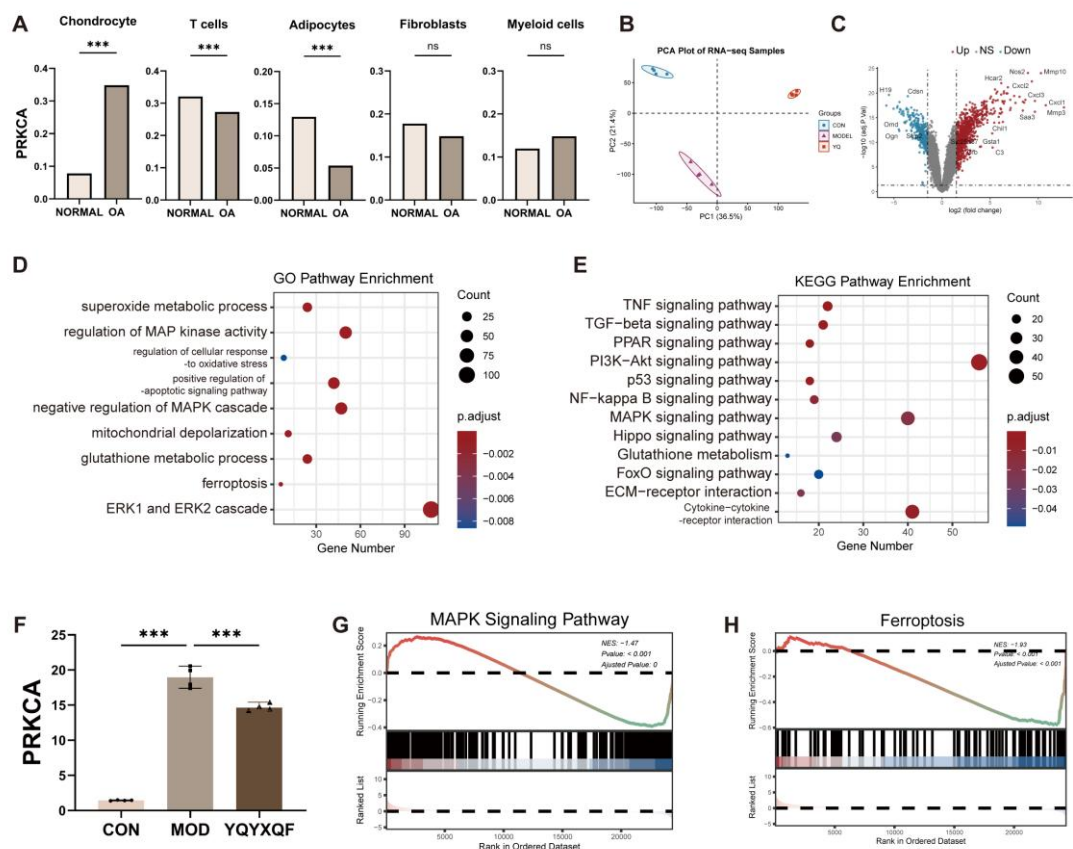
1169 **Figure 8**

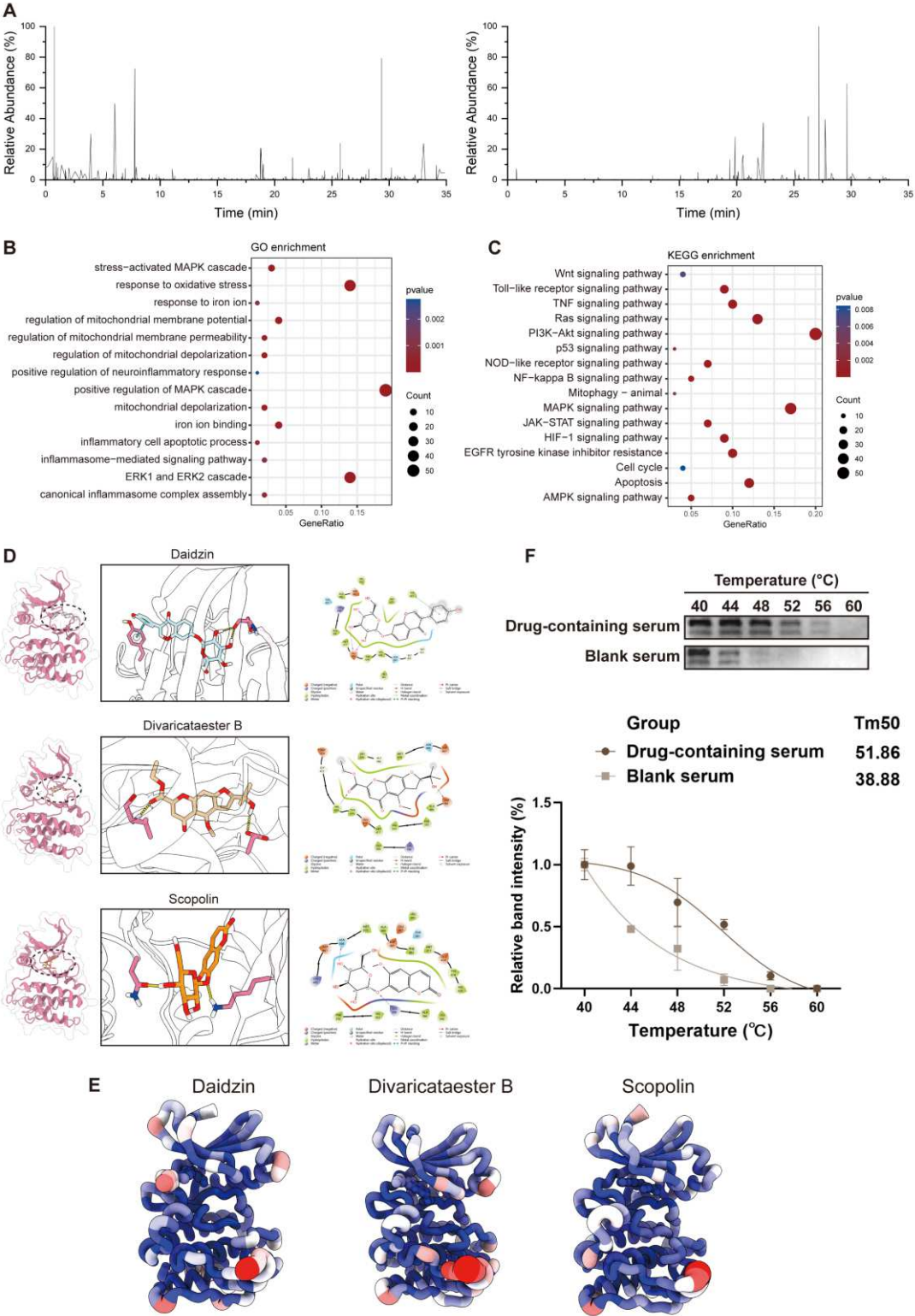


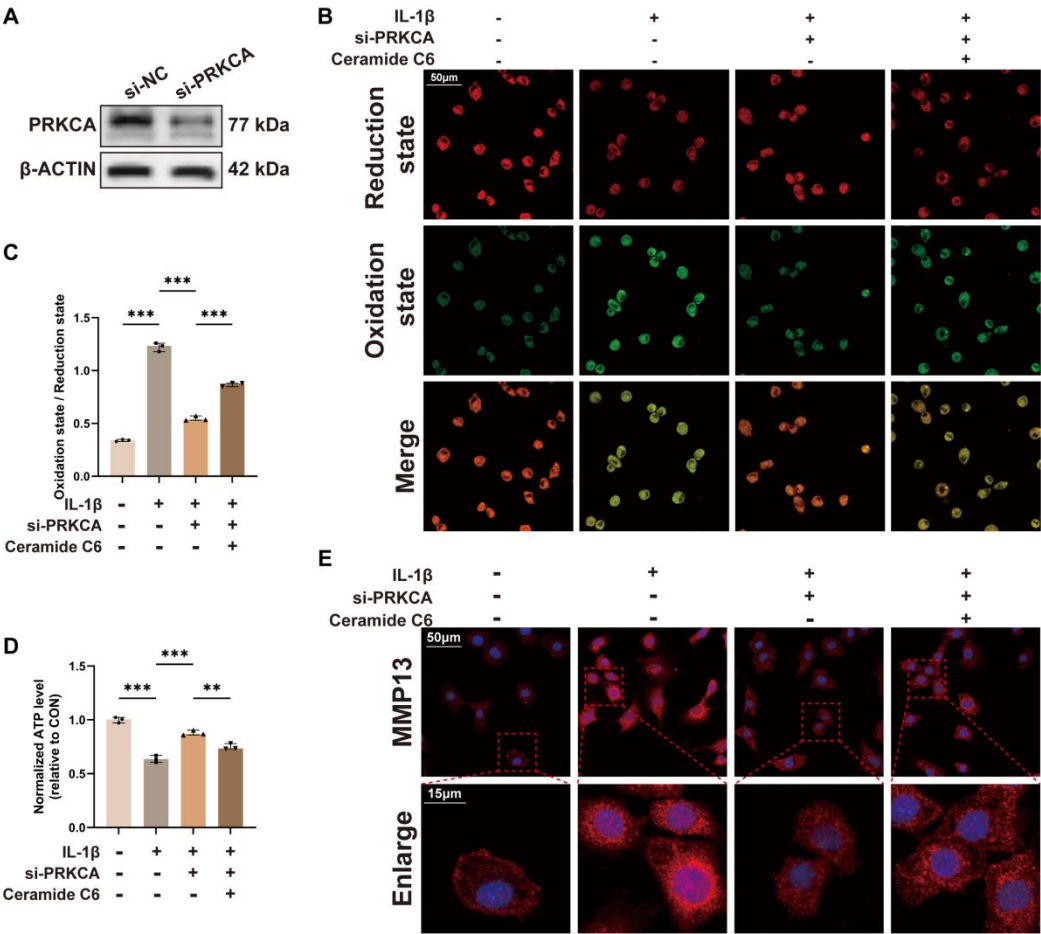
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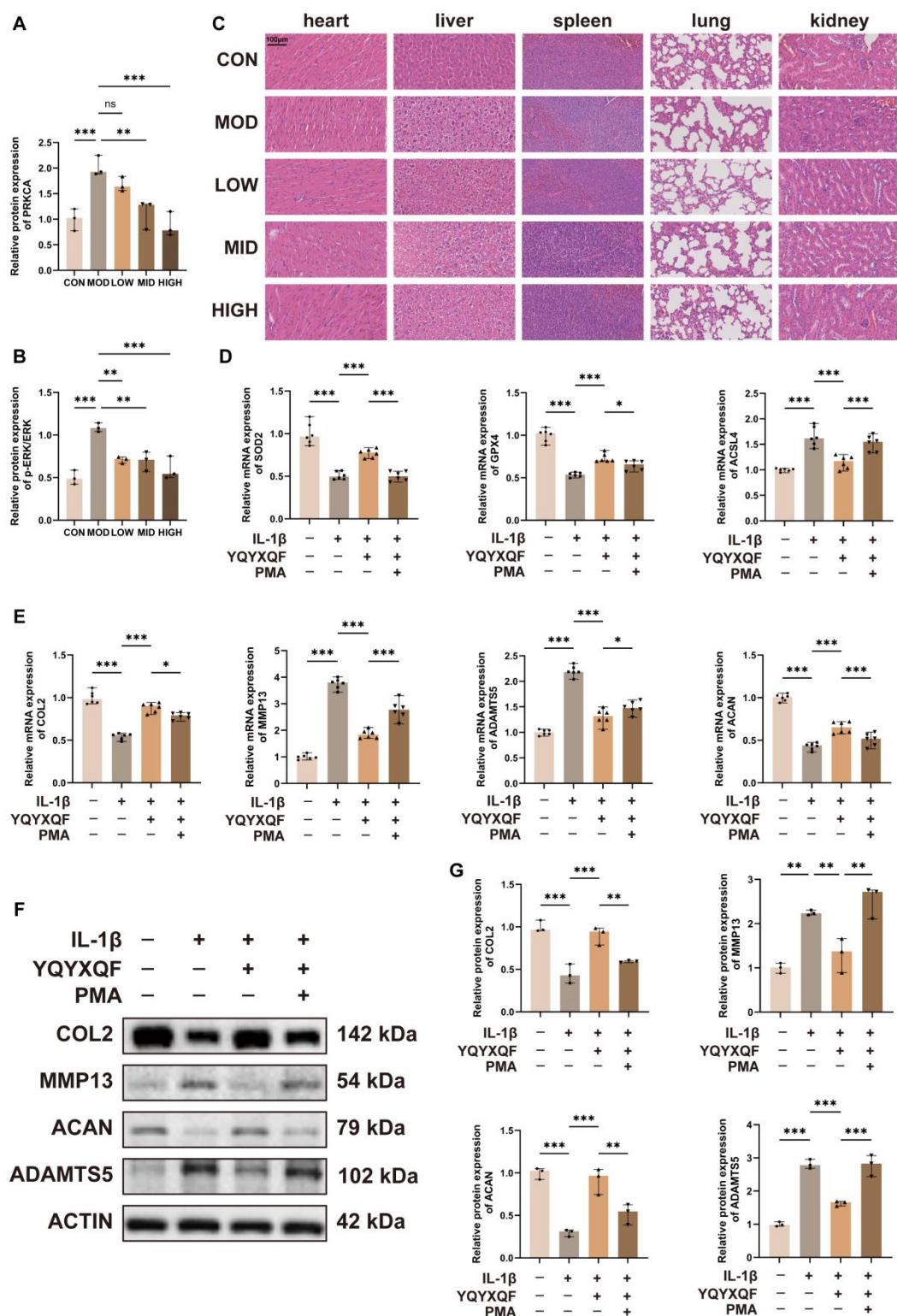
1171 **Figure S1**











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